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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 42 NOVEMBER, 1953 NO. 5

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

An Evaluation of the Preservation of Human Blood Stored in Experimental Plastic Containers. I. In Vitro Studies. Robert L. Wall, M.D., George Shinowara, Ph.D., Bertha Bouroncle, M.D., Nannie K. M. de Leeuw, M.D., and Charles A. Doan, M.D., Columbus, Ohio ---- 665

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Vol. 42, No. 5, November, 1953. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States, its possessions, Pan-American Countries, $12.00 per year; Canada, $13.00; Foreign, $13.00. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 42 NOVEMBER, 1953 NO. 5

CLINICAL AND EXPERIMENTAL

AN EVALUATION OF THE PRESERVATION OF HUMAN BLOOD STORED IN EXPERIMENTAL PLASTIC CONTAINERS

I. IN VITRO STUDIES

ROBERT L. WALL, M.D., GEORGE SHINOWARA, PH.D., BERTHA BOURONCLE, M.D., NANNIE K. M. DE LEEUW, M.D., AND CHARLES A. DOAN, M.D.
COLUMBUS, OHIO

RECENT demands for improved methods of collecting and storing human blood for civilian and military use have produced two experimental plastic containers differing radically from the conventional glass containers now used. In contrast to glass containers, these units have nonwetting surfaces, small volume prior to filling, no need for air vents and, because of the nature of the plastics used, a resilience to shock. While these qualities are desirable in filling both military and civilian requirements, there remains the major concern as to the transfusion value of the blood after storage in these containers. To answer this question a study follows in which human blood was collected and stored in these two types of experimental containers, as well as in the conventional glass container and then subjected to a series of in vitro and in vivo tests. It must be emphasized that this study involves solely a comparison of the effective preservation of blood collected, stored and administered by each of the three methods, i.e., the two plastic containers as compared with the conventional glass container, and *does not* contrast the individual differences in the methods such as wetting versus nonwetting surfaces, differences in anticoagulant solutions, etc.

One of the plastic containers under evaluation consisted of a pliable, nearly transparent plastic bag with a sealed-in rubber-plastic compound top and containing 70 ml. of N.I.H. Formula A* anticoagulant solution.† Sepa-

From the Departments of Medicine and Pathology, Ohio State University, College of Medicine.

This work was supported in part by a grant-in-aid from the National Institutes of Health.
Received for publication, March 31, 1953.

*Sodium citrate 22.0 Gm., citric acid 8.0 Gm., dextrose 24.5 Gm., plus water for injection to 1,000 ml.

†Manufactured by Abbott Laboratories, North Chicago, Ill.

rate donor and recipient sets made of plastic tubing with conventional donor and recipient needles were supplied by the manufacturer. This container is referred to as "container A" throughout these studies.

The other plastic container consisted of a pliable, nearly transparent unit with attached donor tubing and needle, the latter of laminar-flow design and coated to be hemo-repellent. An indwelling plastic filter of large pore size, as well as two outlet areas were on one end of this bag.* The bag contained 75 ml. of N.I.H. Formula A anticoagulant solution and is referred to as "container B" throughout these studies.

The third container was made of glass, capped with a rubber diaphragm; it contained a glass air vent tube, and a vacuum representing the method most widely used in this country today.† It is referred to as "container C" throughout these studies. The anticoagulant solution consisted of 120 ml. of N.I.H. Formula B‡ anticoagulant solution.

PROCEDURE AND METHODS

Six random male donors were selected at the Columbus Regional Red Cross Blood Center. Two were used for each of the three styles of equipment listed above. All containers were precooled in ice baths and were kept in these ice baths during the bleedings and subsequently stored at 4° C. for the duration of the study. Bleedings into containers A and B were accomplished by gravity, while bleedings into containers C were by vacuum.

The containers were sampled aseptically, after uniform mixing, at fourteen hours of storage and at weekly intervals for four weeks thereafter. The quantity of blood removed at these samplings was sufficient for all in vitro tests except the coagulation times, profibrinolysin, and antitrypsin titers for which special sampling equipment was used, necessary for the methods. These blood samples were immediately distributed to the participating laboratories where the individual tests were performed.

It was recognized from the outset that there was no single in vitro method capable of giving an accurate picture of the state of preservation of these bloods. This has been proved by many investigators in the past. However, it was thought that by evaluating these samples of blood with a battery of tests and using each blood longitudinally as its own control, certain differences might be found which, when correlated with in vivo survival studies of similarly treated bloods, would adequately evaluate the preservation of these bloods, and hence any differences which might exist between the three containers. Consequently, twenty-nine in vitro methods were applied to each sample from each container during the storage period.

RESULTS

Tests Based on Morphology.—The total erythrocyte counts, hemoglobin values (photometric oxyhemoglobin method), and hematocrit values only rarely exceeded the inherent errors of the methods in these laboratories. Therefore, there was no significant difference either in a single container during twenty-eight days' storage, between containers of a single type, or among different containers. The survival of transfused erythrocytes in Part II more clearly defines the condition of these preserved erythrocytes.

*Manufactured by Fenwal Laboratories, Inc., Ashland, Mass.

†Manufactured by Baxter Laboratories, Inc., Morton Grove, Ill.

‡Sodium citrate 13.2 Gm., citric acid 4.8 Gm., dextrose 14.7 Gm., plus water for injection to 1,000 ml.

Total leukocytes at fourteen hours' preservation showed a mean of 4,925 with a range of 3,650 to 5,650 per cubic millimeter, and at twenty-eight days a mean of 2,090 with a range of 1,150 to 2,650 per cubic millimeter. While it appeared that there was a slow steady decline in leukocytes, this observation is not true on direct observation of these bloods by supravital staining. The initial observation at fourteen hours showed preservation of the morphology of the leukocytes, but there was great reduction in motility, and many forms showed the excessive Brownian movement characteristic of cell irritability and impending death. There was no difference between the leukocytes of bloods in either A, B, or C containers. The second at one week and subsequent weekly samplings showed no sign of life in the leukocytes.

Platelet counts performed both by Rees-Ecker and Dameshek methods presented great difficulty in making accurate counts, which increased during storage, due to platelet clumping. This bias of chance distribution raised the inherent error of the method to a level which destroys the validity of any count.

Reticulocyte counts performed by Dameshek method varied from 0.2 to 2.0 per cent. While no definite difference was observed between the bloods stored in A, B, and C containers, it is true that reticulocytes were present *as* reticulocytes throughout the preservation period showing lack of maturation in their abnormal in vitro habitat.

Tests Based on Chemistry.—Whole blood pH measured with a glass electrode electrometer showed a mean of 6.64 and a range of 6.8 to 6.5 in all six containers at fourteen hours' preservation. At the end of the twenty-eight days' preservation, the mean was 6.37, with a range of 6.48 to 6.32. The blood samples from type A containers were least acid, while those in type B containers were most acid. The variations in the initial pH values may be attributed to differences in anticoagulant preparations as well as the six individual bloods and the three different containers. All six containers showed significant pH decrease during the twenty-eight day preservation period.

Plasma optical densities at 450 mμ and 540 mμ were determined with a Coleman Junior spectrophotometer. The mean and range of increases in optical densities from the first observations at fourteen hours to the last observations at twenty-eight days were: 540 μ, 0.451 (0.318 to 0.658); 450 μ, 0.498 (0.358 to 0.602). These data indicate the changes were due primarily to increases in hemoglobin concentration. But the differences between individual blood donors *within* container groups approached the differences *between* container groups. Apparently this "spontaneous hemolysis" is entirely related to individual variations between bloods and not to the containers in which they were stored.

Plasma sodium and potassium determinations were performed on a flame photometer. There was a steady decline in plasma sodium during the twenty-eight days of preservation. The mean decrease from fourteen hours to twenty-one days was: container A, 23.9 meq., container B, 26.6 meq., and container C, 22.2 meq. Plasma potassium conversely showed a steady increase in all

bloods. The mean increase from fourteen hours to twenty-eight days of preservation was: container A, 24.02 meq., container B, 17.47 meq., and container C, 17.70 meq. (see Fig. 1).

Plasma chlorides and whole blood chlorides were determined by the method of Schales and Schales,[1] the latter on Folin-Wu protein-free filtrates. These determinations showed no appreciable difference between the bloods of different containers or as a function of the time of storage.

"Blood sugar" measured as total reducing substances by the Folin-Wu method showed variations related to the initial blood sugar level, which in turn are related to the anticoagulants previously described.

Total plasma proteins measured on a filter photometer by the method of Greenberg[2] showed less than 4 per cent variation throughout the storage period. Any differences which did exist in these 30 determinations were related to the initial (i.e., fourteen hour) level and reflected individual donor differences rather than conditions of preservation.

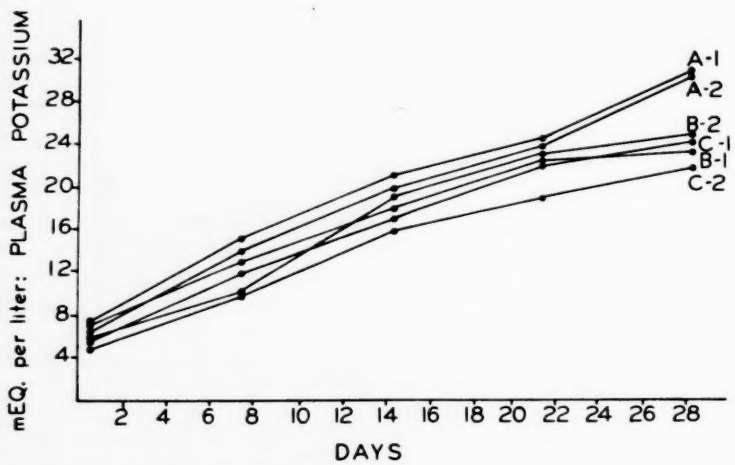

Fig. 1.—Plasma potassium levels of whole blood during a twenty-eight day storage period. (See text.)

Plasma prothrombin concentrations measured by the homologous isolation procedure of Shinowara[3] are recorded in Table I. The mean value found in 51 normal individuals was 81.8 units per milliliter (S.D. ± 14.5).

Table I shows that the plasma prothrombin levels of A-1 and A-2 were well within the normal limits in the initial fourteen hour samples and were essentially the same at the end of twenty-eight days. The prothrombin levels of B-2, C-1 and C-2 were all moderately diminished in the fourteen-hour samples. B-1 did not not show much change a the end of twenty-eight days. However, B-2, C-1, and C-2 had dropped in prothrombin content in twenty-eight days to not more than 60 per cent of the initial values.

Plasma fibrinogen measured as the thrombin-clottable protein of Fraction I by the chemical technique of Shinowara[4] showed less than one standard

TABLE I. PLASMA PROTHROMBIN ACTIVITY IN UNITS PER MILLILITER

CONTAINER NUMBER	TIME ELAPSED IN STORAGE OF BLOOD				
	14 HOURS	7 DAYS	14 DAYS	21 DAYS	28 DAYS
A-1	78.1	90.2	72.9	61.3	71.0
A-2	76.8	73.1	71.5	52.0	69.0
B-1	52.0	61.7	59.3	45.3	49.6
B-2	42.0	39.3	38.0	26.3	28.5
C-1	59.3	50.0	45.1	38.4	34.0
C-2	53.9	52.5	47.2	32.5	32.6

deviation decrease of all bloods as a function of the time of storage. The mean plasma fibrinogen level of 51 normal individuals was 0.26 per cent (S.D. ± 0.05).

Thromboplastic plasma component (TPC) concentrations were determined quantitatively on the nonclottable subfraction of Fraction I by the method of Shinowara,[3] and are recorded in Fig. 2. The normal TPC activity averages 97 units per milliliter, with a range of 17 to 273 units per milliliter.

The lowest TPC activity found in the fresh plasma of a normal individual is 17 units per milliliter. On this basis C-1, C-2, and B-2 and A-2 were above the lowest normal at fourteen hours. Seven days later only C-1 was within the normal limits. After fourteen, twenty-one, and twenty-eight days all the values were below 17 units per milliliter.

Tests Based on Physical and Immunologic Characteristics.—Osmotic fragility of erythrocytes was measured at 32 concentrations of sodium chloride in phosphate buffer at pH 7.4, the supernatant plasma optical densities read on an Evelyn photoelectric colorimeter at 540 μ. This test was repeated on duplicate samples of blood following sterile incubation for twenty-four hours at 37° C. Because of the great number of osmotic fragility tests performed (72 in number) these data must be presented in discussion form.

Before incubation: In general, the trend of the curves obtained, expressing the per cent hemolysis of erythrocytes at each sodium chloride concentration, did not differ strikingly from one container to another. This is particularly true during the first fourteen days of preservation, during which the respective curves progressively shifted to the left. At twenty-one and twenty-eight days of preservation at salt concentrations of 0.5 to 0.8 per cent, the beginnings of the curves are shifted to the left in all cases. But at lower salt concentrations these curves are superimposed or slightly to the right of

TABLE II. OSMOTIC FRAGILITY BEFORE INCUBATION AS PER CENT HEMOLYSIS AT 0.498 PER CENT SODIUM CHLORIDE CONCENTRATION

CONTAINER NUMBER	TIME ELAPSED IN STORAGE OF BLOOD				
	14 HOURS	7 DAYS	14 DAYS	21 DAYS	28 DAYS
A-1	0.2	4.3	11.1	10.7	15.2
A-2	0.7	3.0	6.5	4.0	4.8
B-1	0.1	1.8	3.4	6.3	8.2
B-2	0.2	0.0	4.5	5.3	10.4
C-1	6.7	15.7	37.4	26.7	30.7
C-2	0.9	3.1	10.9	14.7	24.6

the previous determinations. Differences in the bloods of the three containers could best be demonstrated at a sodium chloride concentration of 0.5 (0.498) per cent. (See Table II.)

It can be seen that C container bloods showed an elevated per cent of hemolysis over A and B container bloods. There was no apparent difference between A and B container bloods.

After incubation: In general, the first four determinations, i.e., up to twenty-one days of preservation, showed a progressive shift of the fragility curves to the right. A comparison of pre- and postincubation osmotic fragility curves shows that during the period of preservation the curves gradually approach each other and finally superimpose or cross each other. As the speed of this approach and crossover varied considerably in the individual bloods, this difference is related to the individual blood and not to the container in which it was stored. The phenomenon of crossing of the postincubation curve over the preincubation curve is similar to that seen in most cases of acquired hemolytic anemia.

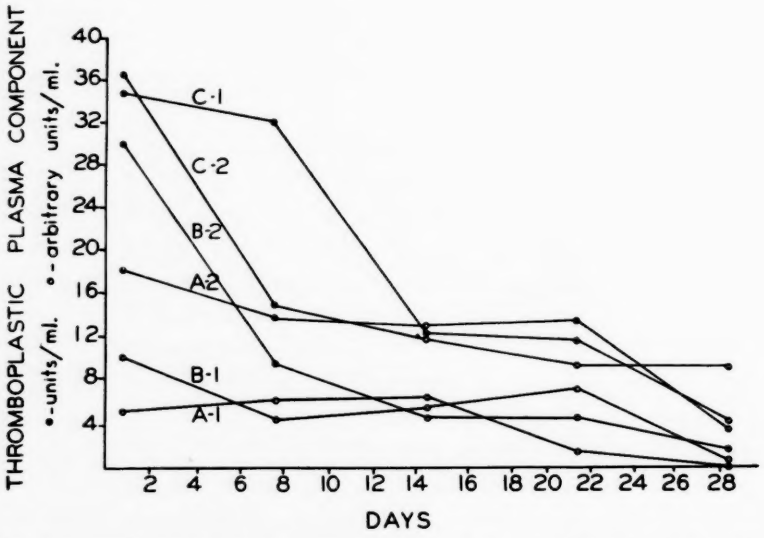

Fig. 2.—Thromboplastic plasma component levels of stored blood. (See text.)

Determinations of the mechanical fragility of erythrocytes before and after twenty-four hour sterile incubation at 37° C. were performed by the methods of Shen, Castle, and Fleming[6] and Shen, Ham, and Fleming.[7] Again differences between bloods *within* a container group approached or exceeded differences *between* container groups. Therefore, these differences are attributable to the individual blood rather than to the containers in which they were stored.

Direct Coombs antihuman globulin tests on the preserved erythrocytes were uniformly negative. Glass surface clotting times performed in triplicate on duplicate blood samples showed a slight prolongation in bloods stored in

A containers (2.5 to 4.5 minutes) during the storage period, whereas no appreciable change took place in bloods from B and C containers. Similarly performed clotting times in silicone-coated test tubes showed no difference to exceed the differences between multiple observations on the same sample of blood.

Profibrinolysin and antitrypsin titers were measured by a method modified from Christensen.[8] These titers were within normal limits for all bloods initially and were unaffected by twenty-eight days of storage.

At each sampling of the containers blood was drawn for bacterial cultures in thioglycollate medium. After incubation for forty-eight hours at 37° C. all these cultures were negative.

TABLE III. SUMMARY OF RESULTS OF IN VITRO TESTS ON BLOOD SAMPLES PRESERVED IN THREE DIFFERENT TYPE CONTAINERS FOR TWENTY-EIGHT DAYS

TESTS	CONTAINERS		
	A	B	C
1. Total erythrocyte count	No change	No change	No change
2. Hemoglobin values	No change	No change	No change
3. Hematocrit	No change	No change	No change
4. Total leukocyte count	Gradual fall with no difference between container groups		
5. Differential leukocyte count	All cells nonviable after initial observation		
6. Platelet count	Cannot be estimated accurately after initial observation		
7. Reticulocyte count	No change	No change	No change
8. Whole blood pH	Least decrease	Greatest decrease	Lesser decrease
9. Optical density plasma—450 μ	Gradual increase in all containers		
10. Optical density plasma—540 μ	Gradual increase in all containers (see text)		
11. Plasma sodium	Decreased	Decreased	Decreased
12. Plasma potassium	Increased (see text)	Increased	Increased
13. Plasma chlorides	No change	No change	No change
14. Whole blood chlorides	No change	No change	No change
15. "Blood sugar"	Decreased at uniform rate from varying initial concentrations		
16. Total plasma proteins	No change	No change	No change
17. Prothrombin concentration	Very slight decrease	Variable decrease	Decrease below 60 per cent initial level
18. Plasma fibrinogen	No change	No change	No change
19. TPC	Rapid decrease	Rapid decrease	Rapid decrease
20. Osmotic fragility	Individual blood sample differences unrelated to containers		
21. Osmotic fragility after incubation			
22. Mechanical fragility	Individual blood sample differences unrelated to containers		
23. Mechanical fragility after incubation			
24. Direct Coombs test	Negative	Negative	Negative
25. Glass surface clotting time	Slight prolongation	No change	No change
26. Silicone surface clotting time	No change	No change	No change
27. Profibrinolysin titers	No change	No change	No change
28. Antitrypsin titers	No change	No change	No change
29. Bacterial cultures	Negative	Negative	Negative

DISCUSSION

Table III summarizes the results of 870 in vitro tests performed on the preserved blood samples. It can be seen that only a few demonstrated differences between blood stored in different type containers.

The slightly better buffering action of blood stored in type A containers is probably more directly related to the preservative solution than to the containers in which the blood samples were stored.

The marked elevation of plasma potassium in bloods stored in type A containers over both B and C containers is found to occur only at the twenty-eight day sampling. This chemical test is frequently used as an index of gross red cell destruction and may well represent an increased red cell destruction in type A containers at twenty-eight days (see Fig. 1).

Prothrombin concentration was significantly decreased in bloods stored in type C containers, well preserved in type A containers, and preserved to a variable degree in type B containers. These observations suggest a valid difference in the bloods stored in the different containers.

Thromboplastic plasma component (TPC) is exceedingly labile in whole blood, stored at 3 to 5° C. The unpublished work of Shinowara's laboratory indicates that TPC can drop as much as 50 per cent six hours after venepuncture in blood similarly collected and stored. Also, there exists a great individual difference in TPC values in normal individuals. However, it may be stated that TPC did appear in greater quantities in blood stored in type C containers than in either A or B containers.

Osmotic fragility tests of preserved erythrocytes is frequently related to the expected in vivo survival of these erythrocytes. While certain individual differences were found to exist in the osmotic fragilities of these preserved erythrocytes at concentrations of 0.5 to 0.8 per cent sodium chloride, these will be related to in vivo survival tests of similarly preserved and transfused erythrocytes in Part II of these studies.

The glass surface clotting times showed a slight prolongation in bloods stored in type A containers; however, this change was of minor degree.

SUMMARY

Twenty-nine in vitro tests were applied to samples of blood from six random donors, each blood drawn and preserved at 4° C. to compare the virtues and limitations of two types of plastic containers versus the conventional glass container. These tests were all applied to each blood sample fourteen hours after bleeding and at weekly intervals for four weeks.

Six of these tests showed differences to exist between the blood samples stored in the different containers. Whole blood pH decreased least in bloods stored in type A containers. Plasma potassium showed a greater increase terminally in bloods stored in type A containers over both B and C containers. Plasma prothrombin concentration decreased least in blood stored in type A containers and most in bloods stored in type C containers. Thromboplastic plasma component decreased at a slower rate in bloods stored in type C containers. Glass surface clotting times were slightly prolonged in bloods stored in type A containers. The evaluation of these differences, related primarily to plasma components, must necessarily end with in vitro tests.

Differences in osmotic fragility of erythrocytes exhibited between the different blood samples were attributed to individual differences in blood

samples except at higher salt concentrations (0.5 to 0.8 per cent). At these concentrations bloods stored in type C containers demonstrated greatly increased hemolysis. This difference is further investigated by in vivo methods in Part II of these studies.

The following personnel are responsible for technical assistance in this study. *Formed elements:* Georgia Gwinner, M.T.; *Optical density, pH, and coagulation components:* Dolores M. Johnston, B.S., M.T., Marilyn J. Herrit, B.S., M.T., Marian M. Starkey, B.S., M.T.; *Electrolytes, protein and sugar:* Donna F. Bitzel, B.S., M.T., Martha I. Walters, B.S., M.T., Margaret A. Delaney, B.S., M.T., Rachel B. Earp, A.B., M.T.; *Mechanical and osmotic fragilities:* Robert D. Palmer, Alvinza E. Bunner, B.A., Audrey Buxton, B.S.; *Recalcified clotting times, profibrinolysin and antitrypsin titers:* Jean H. Smelker, Carl Spragg.

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AN EVALUATION OF THE PRESERVATION OF HUMAN BLOOD STORED IN EXPERIMENTAL PLASTIC CONTAINERS

II. In Vivo Studies

Robert L. Wall, M.D., Nancy M. Buckley, M.D., and Charles A. Doan, M.D.,
With the Assistance of Georgia Gwinner, M.T., and Lilla Sun, M.S.
Columbus, Ohio

MOST investigators in the field of blood preservation believe there is little correlation between present in vitro methods of estimating erythrocyte preservation and in vivo estimates of transfused erythrocyte survival, the latter being the final test of the effectiveness of preservation. As has been demonstrated in Part I of these studies, no one nor any group of the in vitro tests thus far applied has clearly differentiated between the ultimate functional dependability of erythrocytes preserved in either of the two types of plastic containers and in conventional glass containers. With this in mind a series of studies were undertaken to evaluate in vivo survival time of erythrocytes after their preservation in one or the other of these three types of containers for various intervals before being transfused into normal volunteer recipients.

The two plastic containers, one designated "Container A,"* another designated "Container B"† and a conventional glass container for comparison designated "Container C"‡ were identical to those contained in the previous study (see Part I).

METHODS

a. Random donors of blood groups O, Rh-positive (D or Rho) volunteering at the Regional Red Cross Blood Center, Columbus, Ohio, were bled by the three methods until nine donors had been found of blood type N, three bleedings obtained in each type of equipment.

Each container was weighed before and after bleedings to measure the amount of blood subsequently transfused. The collected bloods were stored at 4° C. undisturbed until transfused.

b. Recipient volunteers were chosen among the physically healthy inmates of the Ohio State Penitentiary, with blood groups O, Rh-positive (D or Rho), type M. Three units of blood, one representing each of the three collection methods, were transfused into three matched recipients after fourteen days, three after twenty-one days, and three after twenty-four days' storage. Blood volumes were performed using Evans Blue dye, and 500 ml. of blood was drawn immediately prior to transfusion of all nine recipients. Transfusions were completed in forty-five minutes (± five minutes). Three minutes after the completion of the transfusion a sample of venous blood was drawn to represent *the initial post-transfusion survival* of transfused erythrocytes.

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This work was supported in part by a grant-in-aid from the National Institutes of Health.

Received for publication, March 31, 1953.

*Manufactured by Abbott Laboratories, North Chicago, Ill.

†Manufactured by Fenwal Laboratories, Inc., Ashland, Mass.

‡Manufactured by Baxter Laboratories, Inc., Morton Grove, Ill.

c. Total blood volumes, plasma volumes, and red cell volumes were estimated by the method of Gibson and Evelyn,[1] incorporating Gibson's correction factor for capillary and venous hematocrit differences.[2] The Ashby differential cell agglutination method, as modified by Young,[3] was employed to estimate survival of the transfused erythrocytes, employing the same lot of dry anti-M antiserums throughout the study.

A known amount of blood was drawn from each recipient and a known amount transfused. Blood volumes had been calculated. From these data an estimate of the *expected ratio* of donor to recipient cells was made. The *actual ratio* of donor to recipient erythrocytes was made immediately post-transfusion and throughout the survival period by the differential agglutination method. The immediate post-transfusion difference between the expected and actual ratios of donor:recipient erythrocytes represents the number of erythrocytes not accounted for in the sample of the recipients' venous blood, and therefore presumably destroyed or removed during the *actual time of administration* of the blood.

RESULTS

The survival time of the transfused erythrocytes is plotted as per cent of a theoretical 100 per cent initial survival in Figs. 1, 2, and 3. The initial observations made three minutes after the completion of the transfusions are plotted as the second point on the graphs. Subsequent samples are plotted according to the number of days each was obtained post-transfusion.

Total red cell volumes of recipients, volumes of red cells transfused, expected ratios of these two, and the actual ratios measured by differential agglutination are listed in Table I.

TABLE I. DATA FOR ESTIMATION OF INITIAL SURVIVAL OF TRANSFUSED ERYTHROCYTES STORED FOR FOURTEEN, TWENTY-ONE, AND TWENTY-FOUR DAYS IN EACH OF THREE CONTAINERS. (SEE TEXT.)

SUBJECT	CONTAINER	TIME OF PRESERVATION PRIOR TO TRANSFUSION (DAYS)	TOTAL CELL VOLUME (ML.)	TRANSFUSED CELL VOLUME IN ML.	EXPECTED RATIO OF TRANSFUSED TO RECIPIENTS CELLS	ACTUAL RATIO OF TRANSFUSED TO RECIPIENTS CELLS	PERCENTAGE CELLS LOST DURING TRANSFUSION	PER CENT SURVIVAL IMMEDIATELY POST-TRANSFUSION
C. P.	A	14	2827	212	7.49/100	5.25/100	29.7	70.3
P. H.	B	14	2573	255	9.91/100	8.62/100	13.0	87.0
C. J.	C	14	2265	188	8.30/100	5.96/100	28.1	71.9
R. L.	A	21	2289	180	7.86/100	4.21/100	46.1	53.9
P. L.	B	21	1989	235	11.81/100	9.79/100	17.0	83.0
H. M.	C	21	2478	213	8.59/100	7.04/100	18.5	81.5
L. P.	A	24	2256	210	9.31/100	4.23/100	54.3	45.7
C. N.	B	24	2326	214	9.20/100	4.91/100	46.7	53.3
H. P.	C	24	2265	201	8.87/100	5.49/100	38.3	61.7

Total red cell volumes of recipients had a mean of 2,363 ml. and a range of 1,989 to 2,827 ml. The volumes of transfused erythrocytes had a mean of 212 ml., and a range of 180 to 255 ml. The expected ratios of transfused to recipient red cells had a mean of 9.03 per cent with a range of 7.46 to 11.81 per cent. The actual ratios varied from recipient to recipient according to the age of the preserved blood at the time of transfusion.

As seen in Fig. 1, there was little difference in the survival curves of the three bloods preserved fourteen days before transfusion. Evidence of transfused red cell survival up to one hundred nineteen days was obtained in all

three recipients. Blood preserved in Container C perhaps showed some slight superiority.

Fig. 2 shows the survival curves in three recipients receiving blood preserved twenty-one days before transfusion. Here are revealed both proportionate and length of survival differences. While the red cells from Container C showed a survival curve close to normal survival disappearing only after one hundred twelve days, the red cells from Container B could be identified for only ninety-eight days and from Container A for only seventy-seven days. Between fourteen and ninety-eight days there was also a marked difference in the proportionate representation of donor's cells stored in Container C.

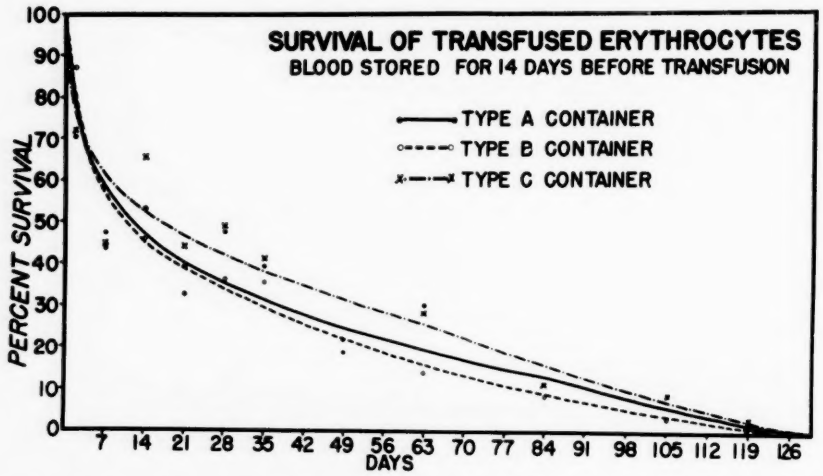

Fig. 1.—Three survival curves of erythrocytes collected and stored for fourteen days in three different types of containers prior to transfusion.

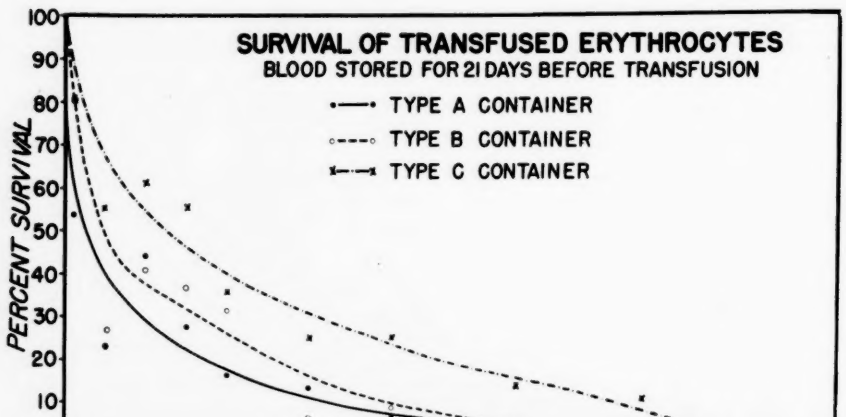

Fig. 2.—Three survival curves of erythrocytes collected and stored for twenty-one days in three different types of containers prior to transfusion.

Fig. 3 shows the survival curves for three units of blood preserved twenty-four days before transfusion to three recipients. The initial observations immediately following completion of the transfusions showed a sharp decrease in surviving circulating donor erythrocytes, varying from 46 (Container A) to 61 per cent (Container C). There is also a marked change in the survival curves. Blood stored in Container C showed a slight reduction in its survival time, 3.5 per cent of transfused cells still present at one hundred five days. Blood stored in Container B showed greatly reduced survival time having only 2.2 per cent cells present at forty-nine days. Blood stored in Container A showed an intermediate pattern, having 0.5 per cent cells remaining at ninety-one days.

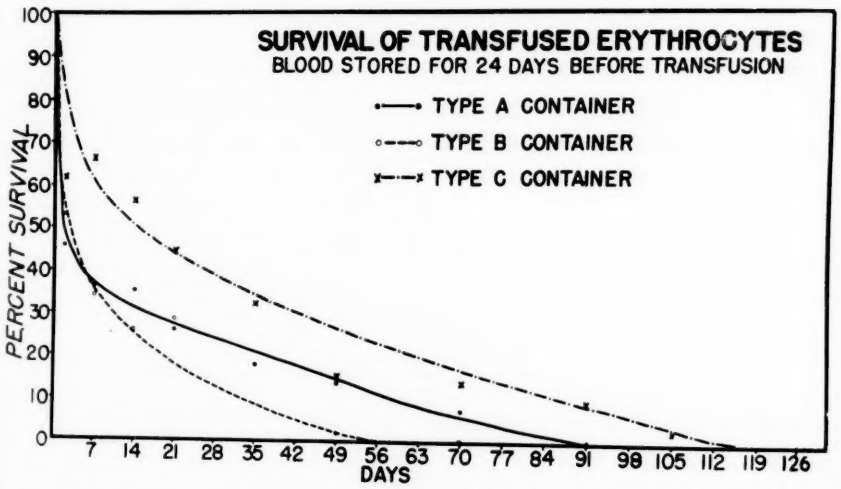

Fig. 3.—Three survival curves of erythrocytes collected and stored for twenty-four days in three different types of containers prior to transfusion.

DISCUSSION

The results of these "pilot" studies show that the estimates of immediate post-transfusion survival of erythrocytes stored for varying periods of time before transfusion appear to vary directly with the length of time of storage. This is most evident in the comparison of blood stored for fourteen days with an average immediate survival of 76 per cent, as compared to that of blood stored for twenty-four days before transfusion with an average immediate survival of 54 per cent.

Gibson and Peacock[4] found a twenty-four hour survival of blood preserved fourteen days to be 96 per cent, and a two hour survival of blood stored for twenty-five days to be 94 per cent. Other reports of twenty-four survivals of blood preserved for fourteen days show 91 and 96 per cent,[5, 6] and for blood preserved twenty-eight days a 60 to 79 per cent twenty-four hour survival.[6, 7] Some of these figures[6] are based on comparisons with fresh blood similarly transfused. These figures are based on blood stored in glass containers.

The difficulty attendant upon the estimation of cell destruction or removal during or shortly after completion of the transfusion has been a major problem to all investigators in this field. The figures here presented show a greater loss of viable cells after storage than most reported but are reported for consideration and evaluation by others.

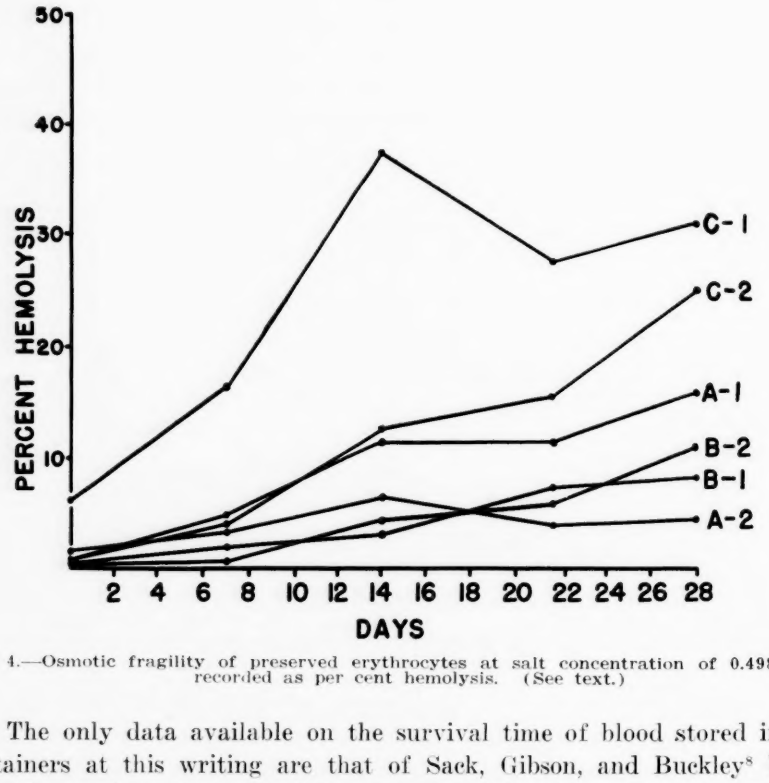

Fig. 4.—Osmotic fragility of preserved erythrocytes at salt concentration of 0.498 per cent recorded as per cent hemolysis. (See text.)

The only data available on the survival time of blood stored in plastic containers at this writing are that of Sack, Gibson, and Buckley[8] based on Fenwal containers (type B of the present study). They report "an immediate post-transfusion survival" of one blood preserved fourteen days as 84 per cent by the Ashby method and of three bloods preserved for the same period as estimated by osmotic fragility tests as 91, 87, and 93 per cent, respectively. The immediate post-transfusion survival of our blood preserved fourteen days in this type container was 87 per cent. Two of their blood samples preserved twenty-one days showed immediate circulating survivals of 54 and 77 per cent by the Ashby method, as compared to 83 per cent in a blood similarly stored in the present study. Two of their bloods preserved for twenty-three and twenty-five days showed an immediate survival of 80 and 67 per cent, respectively, while one of our bloods similarly stored for twenty-four days showed a 53 per cent survival.

No data are available for comparison on survival studies performed on blood stored in type A containers.

The second criterion of viability of transfused erythrocytes is the survival in the circulation *after* the initial period. This is especially important because of the error which exists in the estimates of initial survival. It is generally accepted that under optimal conditions erythrocytes have an in vivo life span of approximately one hundred twenty days. Given a known quantity of erythrocytes from normal donors, stored under optimal conditions for a known period, any reduction in the circulating life span following their transfusion into healthy recipients having previously donated a similar quantity of their own blood, is interpreted as evidence of impaired preservation. The principal variation between bloods in this study, it was planned, would be the containers themselves. Most evidence of the one hundred twenty day life span of the erythrocyte, where made by differential agglutination studies, has been on blood stored in glass containers.

While it is recognized that there are potential individual differences which may affect the survival time of blood preserved under identical conditions, the wide variations in the present survival curves suggest viability differences may exist between erythrocytes stored in glass and in plastic containers and that in the present study the erythrocytes stored as whole blood in the glass containers have shown superior survival times over the blood cells collected and stored in both types of plastic containers available for testing at this time.

Mollison and Young[9] believe there is little if any correlation between in vitro and in vivo tests of erythrocyte preservation. Despite recent evidence to the contrary[8] the present authors agree with this observation. As is shown in Fig. 4, the osmotic fragility recorded as per cent hemolysis at 0.498 per cent sodium chloride concentration shows increased fragility of bloods stored in containers C as compared to containers A and B. However, as shown in this study, the survival times of blood stored in containers C are definitely superior to bloods stored in containers A and B.

CONCLUSIONS AND SUMMARY

Healthy volunteer donors selected at the Regional Red Cross Blood Center, Columbus, Ohio, were used for blood collection utilizing two types of plastic containers for comparison with conventional glass blood collecting units.

These blood donations were transfused into healthy male volunteers at the Ohio State Penitentiary after withdrawing similar quantities of the recipients' blood and the survival time of these transfused erythrocytes was estimated by Young's modifications of the Ashby technique.

In this "pilot" study, erythrocytes stored as whole blood in glass containers exhibited better survival times than erythrocytes stored in either of the two types of plastic containers. There was little difference between the two plastic containers.

In vitro studies performed on bloods similarly treated in Part I of this study show no correlation in evaluation of erythrocytes preservation.

Many potential advantages inherent in plastic type containers for preservation and transportation of blood under special circumstances suggest the

desirability of continuing experimental studies with the newer plastics in the hope that greater assurance of viability of stored blood cells may be obtained giving flexibility to the blood transfusion program.

Acknowledgments.—The authors wish to express their gratitude to Dr. Charlotte Winnemore, Medical Director of the Regional Red Cross Blood Center, Columbus, Ohio, and to Warden R. W. Alvis and his associates at the Ohio State Penitentiary, Columbus, Ohio, for their interest and cooperation in making this study possible.

Addendum.—Approximately fifty transfusions were carried out at the University Hospital with each of the three types of containers in this study to test the relative practical utility of each assembly. These data are not included as many of the individual criticisms of each of the methods have already been incorporated in improved models since the time of this study by the respective manufacturers.

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DIKETOGULONIC ACID AND DIABETES MELLITUS

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DIABETES mellitus has been produced in animals by injecting dehydroascorbic acid.[2] Penney and Zilva,[3] and Borsook and associates[4] have shown that dehydroascorbic acid is transformed into either ascorbic acid or diketogulonic acid when it is injected into animals (Fig. 1). The latter reaction prompted us to consider the possible relationship of diketogulonic acid to diabetes mellitus. Accordingly, the following survey was carried out to ascertain whether diketogulonic acid was present in unusual quantities in the blood of human diabetics.

METHODS

The following modification of the methods of Penney and Zilva[6] and Roe and coworkers[5] for the determination of diketogulonic acid was developed for the estimation of this compound in blood specimens:

Reagents.—2,4-Dinitrophenylhydrazine Reagent: 2 Gm. of solid is dissolved in 100 ml. of 9N H_2SO_4 (75 ml. of water + 25 ml. H_2SO_4). After filtering, it is preserved in a dark bottle.

85 per cent Sulfuric Acid: To 100 ml. of distilled water is added 900 ml. of concentrated H_2SO_4.

5 per cent Metaphosphoric Acid: 5 Gm. of HPO_3 dissolved in 100 ml. of water (made up the day it is to be used).

10 per cent Stannous Chloride.—10 Gm. of $SnCl_2$ in 100 ml. of water (made up the day it is to be used, and mixed well immediately before measuring).

Preparation of Filtrate.—15 ml. of 5 per cent metaphosphoric acid was poured into a 25 ml. graduated cylinder. 1.25 ml. of 10 per cent stannous chloride solution was added and the volume made up to 20 ml. with 5 per cent metaphosphoric acid and thoroughly mixed. This precipitating mixture was then transferred to a 125 ml. Erlenmeyer flask. Five milliliters of whole oxalated blood which had been drawn within the hour was introduced in a dropwise fashion with continuous shaking to assure a fine suspension. The solution was then filtered through a smooth, close texture filter paper (Aloe No. 42710) to obtain a water clear filtrate.

Analytical Procedure.—Four milliliter aliquots of the filtrate were added to each of three tubes. One was held as a blank, and to the others was added 1 ml. of dinitrophenylhydrazine reagent. These tubes were rotated gently to mix and were then placed in a 38° C. constant temperature water bath for exactly forty minutes. Since in forty minutes no interference from dehydroascorbic acid was found, the H_2S separation was omitted. The tubes were then transferred to a beaker of ice and water to stop the reaction. One milliliter of dinitrophenylhydrazine reagent was then added to the blank, and while all of the tubes remained in ice water, 5 ml. of 85 per cent sulfuric acid was dropped slowly into each and mixed well with a glass rod. The tubes were then placed in a rack at room temperature for at least thirty minutes before reading. The color is stable for twelve hours.

Received for publication, April 17, 1953.

Using the blank as a reference, samples were read in a Coleman Universal Spectrophotometer at 550 mu (without using the 14-214 filter supplied with the instrument). Values were interpolated from a transmittance curve as prepared from standard solutions of diketogulonic acid as described below.

Calibration Curve.—2,3-diketogulonic acid was prepared by the bromine oxidation method of Roe and Oesterling.[5] A weighed quantity of *l*-ascorbic acid was placed in a volumetric flask, dissolved in a small amount of 5 per cent HPO_3, and 0.5 per cent solid $SnCl_2$ added. Bromine water was added until the oxidation was completed and the excess bromine removed with CO_2. Appropriate dilutions were made from this stock solution (made up to volume) so that standards ranging from 1 to 10 gamma per 4 ml. are obtained. Duplicate 4 ml. aliquots of each strength of diketogulonic acid were then treated exactly as described above to develop the characteristic color. Tubes were read at 550 mu using a reference standard to which dinitrophenylhydrazine was not added until after the incubation period. When the values obtained were plotted on semilog paper, a straight line bisecting the origin was obtained.

Calculations.—D=dilution of blood. C=concentration of diketogulonic acid in 4 ml. of blood filtrate (from the transmittance curve). $\frac{C}{4} \times D \times 100$=concentration of diketogulonic acid in 100 ml. of blood. $\frac{C}{4} \times 500$=gamma DKA/100 ml. blood.

Fasting blood specimens were secured from normal individuals and diabetics and compared for their diketogulonic acid content.

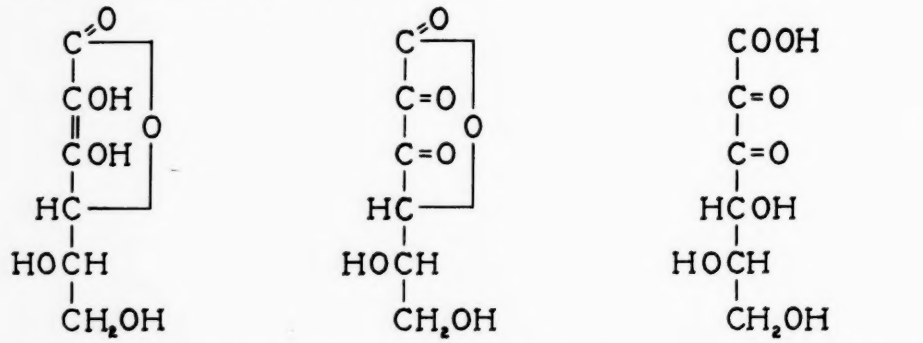

Fig. 1.

RESULTS

Nondiabetic Blood.—The blood of 27 nondiabetics was examined for diketogulonic acid. None was found to contain any diketogulonic acid.

Diabetic Blood.—The blood of 28 proved diabetics was similarly tested. All contained some diketogulonic acid. The values ranged from 25 to 650 gamma per 100 ml. of blood. Four of these diabetics who were controlled with only dietary management had a range of 25 to 137.5 gamma diketogulonic acid per 100 ml. of blood. Twenty-four of the diabetics using insulin had a range of 31.2 to 650 gamma of diketogulonic acid per 100 ml. of blood (Table I). The quantity of diketogulonic acid in the diabetic blood had no relationship to the level of the blood sugar in a given individual although rather wide variations were noted from time to time. (Table II.)

TABLE I. DIKETOGULONIC ACID CONTENT OF THE BLOOD OF DIABETICS.

PATIENT'S NO.	AGE	DURATION	BLOOD SUGAR	URINE SUGAR	GAMMA DKA/100 ML. BLOOD	INSULIN
520517 W.	58 yr.	New	205	1+	75.0	No
520533 C.	36	2 wk.	156	0	25.0	No
520093 W.	50	7 mo.	120	0	62.5	No
510560 F.	60	6 yr.	72	0	137.5	No
520435 W.	59	2 mo.	172.5	0	312.5	Yes
510030 J.	62	5 yr.	130	0	125.0	Yes
520597 T.	53	18 yr.	187.5	0	31.2	Yes
520580 C.	39	14 yr.	225	4+	100.0	Yes
510035 L.	62	6 yr.	165	0	312.5	Yes
510855 L.	59	6 yr.	200	0	237.5	Yes
520520 V.	32	2 wk.	237.5	4+	162.5	Yes
520463 B.	53	13 yr.	237	4+	162.5	Yes
510382 M.	49	6 yr.	232	4+	162.5	Yes
510448 M.	43	29 yr.	250	4+	562.5	Yes
510894 H.	28	2 yr.	274	0	650.0	Yes
530106 O.	50	1 yr.	155	0	162.5	Yes
530117 J.	58	9 yr.	300	2+	625.0	Yes
530116 C.	59	1 yr.	274	4+	300.0	Yes
510335 D.	64	6 yr.	62.5	2+	437.5	Yes
511015 C.	65	17 yr.	187.5	0	175.0	Yes
510305 C.	72	12 yr.	217	4+	175.0	Yes
510944 G.	38	15 yr.	112.5	0	87.5	Yes
520387 P.	72	19 yr.	217.5	4+	187.5	Yes
520124 D.	42	17 yr.	330	4+	237.5	Yes
520547 S.	14	5 yr.	335	4+	312.5	Yes
520473 J.	60	2 yr.	97.5	0	450.0	Yes
520672 T.	64	5 yr.	177.5	0	650.0	Yes
530064 P.	51	2 yr.	150	0	525.0	Yes

TABLE II. VARIATION IN QUANTITY OF DIKETOGULONIC ACID IN BLOOD OF DIABETIC PATIENTS

PATIENT	DATE	BLOOD SUGAR	GAMMA DKA/100 ML. BLOOD
510030 J.	10/ 9/52	167.5	187.5
	10/ 9/52	130.0	125.0
520520 V.	10/11/52	237.5	162.5
	2/23/53	70.0	675.0
510855 L.	2/24/53	150.0	262.5
	10/ 7/52	200.0	237.5
520672 T.	3/24/53	146.0	237.5
	2/24/53	177.5	650.0

SUMMARY AND CONCLUSIONS

Utilizing an original modification of existing procedures diketogulonic acid was estimated in the blood of normal subjects and diabetic patients. None was found in 27 nondiabetics examined, but substantial quantities of diketogulonic acid or a closely related chromogen were found in all of the diabetics. The quantity determined seemed to have no relationship to the level of blood glucose or to whether or not the patient was receiving insulin. These results are admittedly preliminary, but do bring to mind a possible relationship of diketogulonic acid or dehydroascorbic acid to diabetes mellitus as seen in man.

Clinical material supplied by H. E. King, M.D., and A. J. Gabriele, M.D.

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EFFECT OF DIALIZED CONCENTRATED HUMAN URINE ON RENAL FUNCTION IN THE HUMAN BEING

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LITTLE[1, 2] has on two occasions reported that dialized and concentrated human or dog urine, when injected intravenously into dogs, causes a marked diuresis. The finding of a diuretic factor in normal human urine is somewhat contrary to current concepts of renal physiology, but the theoretical and clinical implications are obvious. The mechanism of action has not been demonstrated, though a possible clue is given by the work of Green and associates,[3] who demonstrated that similar preparations when injected intra-arterially into the legs of dogs give a marked vasodilation and increased blood flow as measured by an electromagnetic flow meter. The changes in renal plasma flow, glomerular filtration rate, and filtration fraction have not been studied. This paper records an attempt to extend the findings of Little to man in order that fuller investigation of renal function could be made.

MATERIALS AND METHODS

The methods of collecting, dializing, and concentrating the urine are, with the exceptions noted, exactly those employed by Little. Urine was voided by the author directly into sterile cellophane dialysis bags using as sterile precautions as possible; the first portion of the urinary stream was not collected. The bags were sealed by tying with string and were immediately placed in a large dialysis chamber filled with cold running tap water. Dialysis was carried out in the refrigerator at 5° C. for forty-eight hours against running tap water, and for an additional twenty-four hours against running distilled water. At the end of the seventy-two hours of dialysis the bags were removed and placed in a current of warm air from a large fan. After being exposed to this current of warm air for about twenty-four hours the concentrated residue was aseptically removed with a sterile needle and syringe and placed in a plastic bottle in a deep freeze unit. Each day additional concentrate was added to this bottle and over a period of two weeks a total of ten liters of urine was dialized and concentrated to a volume of 250 ml.; this represents a forty fold concentration. The concentrate showed only a trace of chloride; a portion was evaporated to dryness and the solid content was found to be 291 mg. per 100 ml.

For the further experimental work the author served as subject. Renal plasma flow was determined by the subcutaneous injection of 5 per cent para-aminohippurate, and the glomerular filtration rate was determined by endogenous creatinine and allantoin as previously reported for this subject.[4] The experiment was conducted as follows: three liters of tap water were drunk over a period of forty-five minutes to provoke a degree of diuresis which would enable the collection of 50 to 100 ml. urine samples at five minute intervals for PAH, creatinine, and allantoin determinations. In the first experiment as a control 30 ml. of saline was injected intravenously about forty minutes after the last water had been drunk. In the second experiment, instead of injecting saline, 30 ml. of the urine concentrate were injected. The subsequent happenings are recorded below and illustrated in Table I.

Received for publication, May 22, 1953.
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TABLE I. SHOWING THE EFFECT OF INJECTION OF SALINE AND CONCENTRATED URINE DIALYSATE ON URINE FLOW IN THE HUMAN BEING.

MINUTES AFTER DRINKING LAST OF THE WATER	30	35	40	45	50	55	60	65	70	75	80
Urine flow in ml. per minute											
(a) control	16	16*	16	15	15	14	12	12	10	10	9
(b) experiment	16	16†	0.6	No further urine was obtained for two days							

*30 ml. saline injected at the end of this collection period.
†30 ml. concentrated urine injected at the end of this collection period.

RESULTS

The urine flow resulting from the drinking of the water reached a maximum of sixteen milliliters per minute and was sustained at this rate for about thirty-five minutes. Renal plasma flow was found to be 695 ml. per minute, and glomerular filtration rate by creatinine and allantoin to be 121 and 123 ml. per minute, respectively. The filtration fraction was 17.5 per cent. None of these values were changed by the injection of saline (Table I).

The injection of 30 ml. of urine concentrate was made immediately after a five-minute period of urine collection. This produced unexpected results. Immediately and for about half a minute after injection of the urine concentrate a warm sensation was felt in the vein. About a minute later a warm sensation was felt in the lungs, this containued for about two minutes. For the next three minutes nothing unusual was experienced, but at the next collection period only three milliliters of urine was obtained; presumably this amount was already in the tubules, kidney pelvis, and ureter. The quantity was too small for analysis. This immediate renal shutdown continued so that there was a complete cessation of urine flow not only for the remaining forty-five minutes of the intended experiment, as shown in Table I, but also for the next two days. About five minutes after the injection a slight headache began, the skin became white, the hands and feet were cold, perspiration occurred all over the body, and rigors set in which could be controlled only by voluntary acts such as walking. The blood pressure rose from 130 to 200 mm. Hg, while the heart action increased from 65 to 100 beats per minute. Respiration became a conscious act, although it was not essentially altered. This state was maintained for about twenty minutes, and then, just as the symptoms were becoming less severe, a sudden attack of vomiting occurred. About an hour later marked diarrhea set in. At this time the blood pressure and heart rate began to return to normal over a period of about thirty minutes. As this was taking place a slight temperature of 100 to 101° F. became apparent. This temperature and the sweating continued for about two days, as did the vomiting, diarrhea, and headache. Over these two days the only form of therapy was seven liters of intravenous saline. During this time there was a great thirst with a craving for salty tomato juice; however, any fluids taken by mouth were immediately regurgitated. No biochemical determinations were done. At the end of the two days these symptoms disappeared during a period of about eight hours. The total anuria continued until the beginning of the third day when a small amount of dark brown urine was passed; the urine had a specific gravity of 1.036, but there was no protein or blood. By the end of the third day the specific gravity was normal and the volume varied directly with the oral intake of water. On the fourth day the urine became smoky with large quantities of red blood corpuscles without hemolysis. This hematuria persisted for the next thirty-six hours, at the end of which time it suddenly disappeared.

Studies carried out one month later revealed a normal renal plasma flow and glomerular filtration rate, and up to one year later no hematuria or proteinuria has been noted.

DISCUSSION

Although the results of this experiment fail to demonstrate the presence of a diuretic factor in man, they do not thereby disprove its existence.

There is no reason to doubt that the present dialysate contained a diuretic factor(s). Little has reported that not all persons have the diuretic factor in their urine. However, the author, working in Little's laboratory, has made preparations of his own urine in exactly the manner as those used in this experiment. On injection into dogs, the preparations produced the same degree of diuresis as was reported by Little.

Any possible diuretic effect in man was, however, completely inhibited by the gross vasomotor disturbance. This disturbance was presumably due to the presence of a toxic substance(s) in the relatively unrefined urine concentrate. Such a substance produces a severe adverse reaction in man but does not usually have this effect in the dog, although Little has noted that occasionally dogs will show some untoward reaction such as defecation and temporary decrease in urine flow.

The nature of the reaction in man appears to be a transient vasoconstriction in the renal cortex and irritation of the alimentary tract, with vasoconstriction elsewhere in the body. This would account for the hypertension. Analogous symptoms such as sudden vomiting, diarrhea, and a period of anuria followed by hematuria occur in a number of cases of renal cortical necrosis not associated with obstetrics.[5] This condition is clearly related to a local vasoconstriction. There are several known toxins capable of producing renal cortical necrosis. In the present experiment some vasoconstrictor substance of similar type appears either to have been present in the original urine or to have been produced in the preparation of the concentrated dialysate. While it did not produce permanent renal insufficiency it is sufficiently toxic to preclude studies of the effect of unrefined dialysates in the human being.

SUMMARY

Crude dialized and concentrated human urine contains a substance(s) which when injected intravenously into a human being caused marked gastrointestinal upset, headache, transient hypertension, temperature, and complete renal shutdown. Similar preparations give a diuresis when injected intravenously into dogs.

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RELATION OF ACUTE CHANGES IN COLLOIDAL OSMOTIC PRESSURE TO BODY WEIGHT IN PATIENTS WITH HEPATIC CIRRHOSIS AND ASCITES

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A REDUCTION in the concentration of serum proteins,[1] particularly the albumin fraction,[2] and lowering of the colloidal osmotic pressure[3, 4] exerted by these proteins has been claimed to be correlated with the presence or absence of ascites occurring in chronic liver disease. According to this concept, ascites is formed by transudation of fluid from the plasma due to the reduction in colloidal osmotic pressure exerted by the lowered concentration of serum proteins with resultant edema, as dictated by the Starling[5] principle.

Recent observations[6, 7] indicate that factors other than colloidal osmotic pressure may be concerned with the production of ascites in cirrhosis of the liver. Ralli and co-workers[8] have noted an antidiuretic factor in the urine of patients with cirrhosis. Faloon and co-workers[9] observed a decrease in the rate of formation of ascites following a restricted sodium intake, and fluid retention following the administration of sodium. This relationship held true despite the maintenance of serum albumin levels near normal by the infusion of serum albumin. Evidence for specific impairment of renal mechanisms for excretion of sodium has been noted in edematous[10, 11] and nonedematous patients[12] with cirrhosis of the liver.

In the course of investigating the effects of a cation exchange resin on patients with portal cirrhosis,[13] major fluctuations of edema and ascites presented an opportunity to test the relationship between serum protein concentrations and variations in the degree of ascites and edema.

METHODS

Nine patients with ascites, edema, and histologically proved portal cirrhosis were studied before, during, and after administration of a cation-anion exchange resin (Carbo-Resin®). The details are reported elsewhere.[13]

One hundred and one paired determinations of body weight and serum proteins were made at intervals of one to three weeks. Unclothed weights were determined on balance scales at the same time of morning. Blood was drawn without stasis after a period of rest in the sitting position. Serum proteins were determined by the method of Wolfson and Cohn.[14] The osmotic pressure was calculated by the formula[15]: $\pi = 60.9A_w + 22.9G_w - 50$, where π is calculated osmotic pressure in millimeters of water, and A_w and G_w the serum concentration of albumin and globulin, respectively, expressed in grams per 100 ml. of serum, corrected for water content by the formula: $W = 98.40 - 0.718P$, developed by Eisenman, MacKenzie, and Peters.[16]

The regression equations for data relating changes in body weight to changes in total serum protein, serum albumin, and colloidal osmotic pressure were derived,[17] and

From the Institute for Medical Research, University of Louisville School of Medicine.
Aided by grants from Eli Lilly and Company, Indianapolis, Ind., and Kentucky Research Commission.
Received for publication, June 1, 1953.

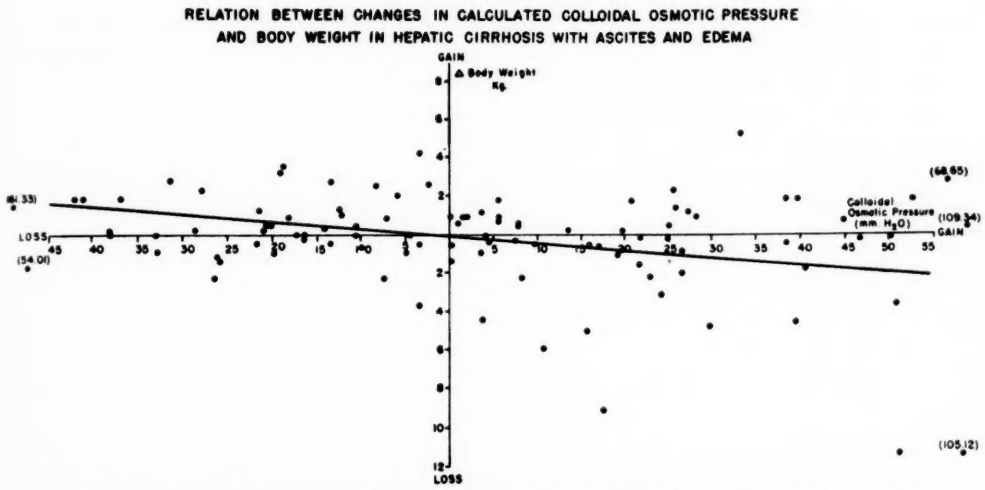

Fig. 1.—Change in edema as determined by body weight change plotted against change in colloidal osmotic pressure. The calculated regression is indistinguishable from that expected on the basis of chance alone ($P < 0.10$ and > 0.05).

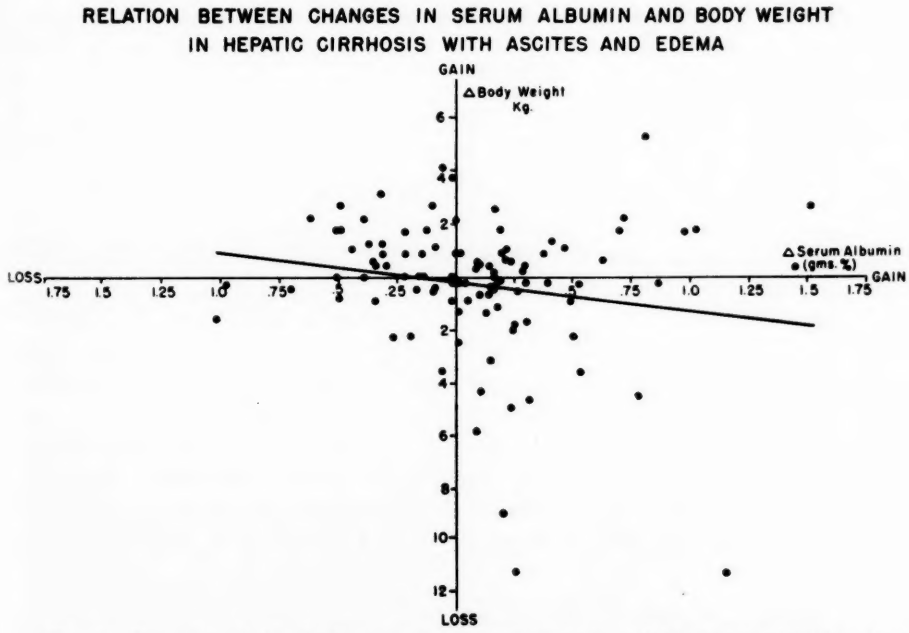

Fig. 2.—Change in state of edema as determined by body weight change plotted against change in serum albumin. The regression line does not differ from that expected on the basis of chance ($P < 0.50$ and > 0.10).

statistical significance tested by means of the "t" test, with fiducial limits at the 5 per cent point, and degree of freedom equal to N–2.

RESULTS

(A) *Relation of Changes in Colloidal Osmotic Pressure, Serum Albumin, and Total Protein to State of Edema.*—Fig. 1 presents a plot of values repre-

senting acute change in body weight versus change in calculated colloidal osmotic pressure. The calculated regression coefficient was indistinguishable from that expected on the basis of chance alone ($P < 0.10$ and > 0.05). Similarly, Fig. 2 shows relation of changes in values of serum albumin to those of acute changes in weight ($P < 0.50$ and > 0.10). Fig. 3 reveals a somewhat closer correlation between change in total protein and state of edema ($P < 0.05$ and > 0.02).

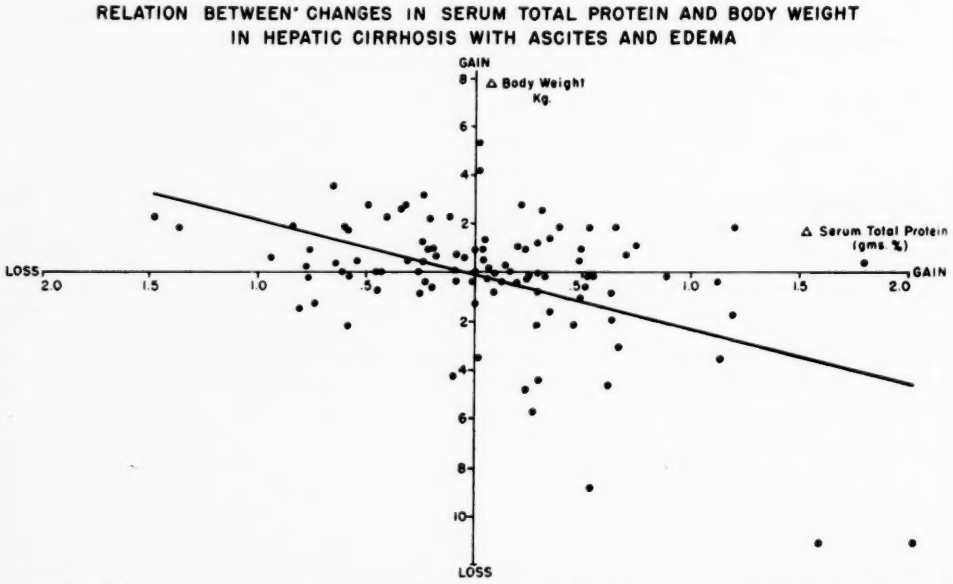

Fig. 3.—Relationship between change in body weight and total serum protein ($P < 0.05$ and > 0.02).

(B) *Relationship Between Body Weight and Colloidal Osmotic Pressure in Each Individual Patient.*—Concurrent changes in body weight and colloidal osmotic pressure of the nine cirrhotic patients observed herein are depicted in Fig. 4. Frequently, body weight may be observed to fall during the early phase of treatment, which is accompanied by loss of edema and ascites, and to increase gradually after the first four to six weeks, presumably due to improvement in the state of nutrition. Colloidal osmotic pressure rose slowly in all patients during treatment, usually lagging behind the loss of ascites and edema.

DISCUSSION

The failure to demonstrate a correlation between short-term changes in serum colloidal osmotic pressures and variation in the degree of edema and ascites in patients with portal cirrhosis may have at least two explanations.

Serum protein concentrations show considerable physiologic variation.[15] The assumption of the upright posture or strenuous exercise will induce hemoconcentration, with an 8 per cent average increase in serum proteins as compared to the supine basal state. Apparently spontaneous variations up to 4.5 per cent have been observed.

Although utmost care was exercised in procurement of samples of serum, unrecognized variations in the state of the patient may have occurred, and the associated fluctuations in serum proteins obscured a relationship between changes in colloidal osmotic pressure and gain or loss of edema.

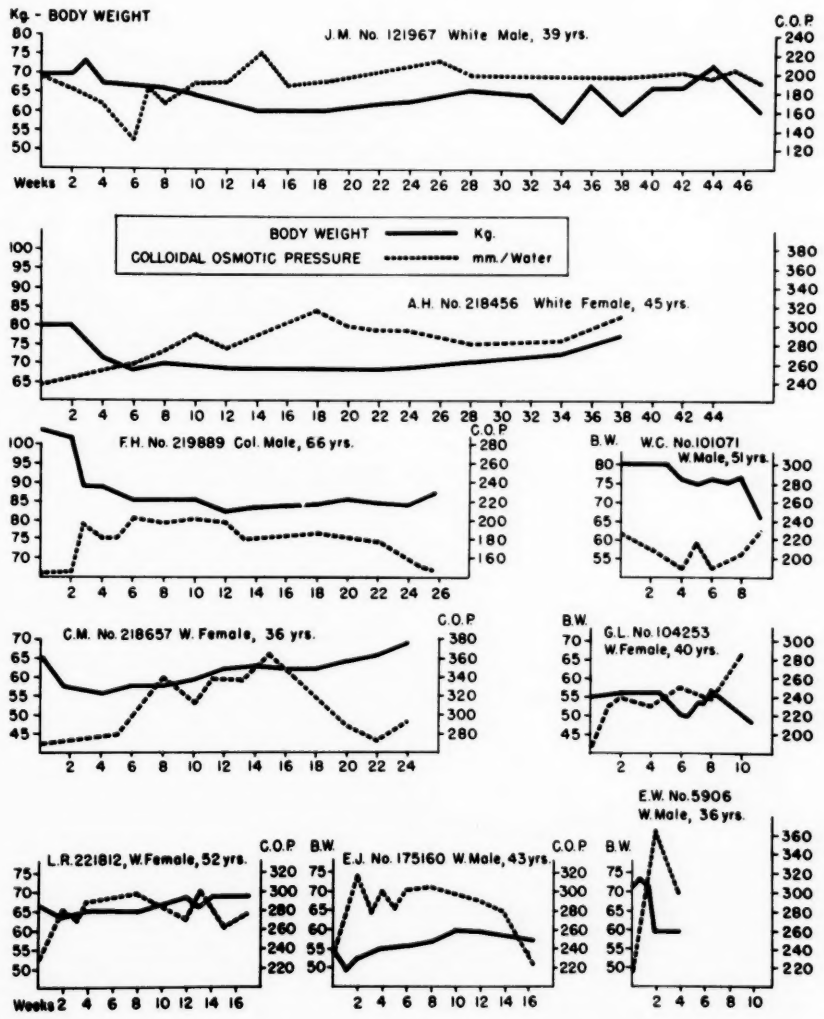
Kg. - BODY WEIGHT
C.O.P.
J.M. No. 121967 White Male, 39 yrs.
Weeks
BODY WEIGHT Kg.
COLLOIDAL OSMOTIC PRESSURE mm./Water
A.H. No. 218456 White Female, 45 yrs.
F.H. No. 219889 Col. Male, 66 yrs.
B.W.
W.C. No. 101071 W. Male, 51 yrs.
C.M. No. 218657 W. Female, 36 yrs.
G.L. No. 104253 W. Female, 40 yrs.
E.W. No. 5906 W. Male, 36 yrs.
L.R. 221812, W. Female, 52 yrs.
E.J. No. 175160 W. Male, 43 yrs.

Fig. 4.—Relationship between body weight and colloidal osmotic pressure in individual patients during periods of observation described in Fig. 1.

It appears likely, however, that factors other than serum protein changes are responsible for the short-term accumulation or dissipation of ascites and edema in patients with portal cirrhosis. That one such factor is electrolyte balance is suggested by the elimination of edema and ascites incident to resin therapy.

The long-term rise in serum proteins and colloidal osmotic pressure are perhaps explained on the basis of improved nutritional states and greater activity by the patient after the loss of edema and ascites.

SUMMARY

Calculated regressions for acute (one to three weeks) changes of body weight versus changes in oncotic pressure and serum albumin in patients with decompensated hepatic cirrhosis were indistinguishable statistically from those expected on the basis of chance alone.

Physiologic variations in serum proteins may have obscured a causal relationship between changes in serum proteins and accumulation or dissipation of edema and ascites, but it appears from the data that factors other than serum protein changes alone govern accumulation and dissipation of edema and ascites in portal cirrhosis.

Acknowledgment.—We are indebted to Dr. William A. Brodsky and Dr. Charles H. Duncan for aid in statistical analysis of the data. The charts were prepared by the Department of Visual Education.

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THE STABILITY OF THE VIRAL POTENCY OF SMALLPOX VACCINE WHEN PREPARED IN CALF GLOBULIN, DEHYDRATED, AND STORED WITHOUT REFRIGERATION

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THE need very often arises to transport and use smallpox vaccine in the field of tropical and subtropical climates where refrigeration is not available. The glycerinated vaccines do not lend themselves for such emergency use because of the rapid deterioration they undergo at temperatures higher than 5° C. The deterioration effect was demonstrated ably by Green[1] who found that these vaccines are almost totally inactivated after twelve days at 37° C.

The need has long been recognized, therefore, for obtaining a vaccine whose potency remains stable sufficiently long to pass the standards of infectivity such as set by the National Institute of Health after exposure to relatively high temperatures for at least thirty days. This period of time was chosen as the criterion of minimal durability because in thirty days' time, it is felt, stocks of vaccine could be transported to distant areas by air and still leave sufficient time for their use in the field.

Numerous attempts have been made to produce this desirable product. Protective colloids of many types have been employed for preparing the virus pulp emulsion. They have demonstrated substantial protection for the virus for limited periods of time when stored at temperatures higher than 5° C. Goodpasture and Buddingh[2] demonstrated that rabbit serum and Buddingh[3] that beef serum has a protective action for the smallpox virus when stored at high temperatures. Buddingh[4] compared the protective action afforded the virus by rabbit and beef serums with that of other substances and found the serums to be the best. Lloyd and Mahaffy[5] diluted tissue-culture virus with equal parts of normal horse serum, and reported that the virus was protected sufficiently to allow it to withstand a temperature of 28° C. for a period of sixty-nine days with only slight deterioration effect. At 37° C., however, it lost potency rapidly after the fourteenth day of storage.

To date the best promise of finding a method of preparing a reasonably durable product has been given by the dehydrated vaccines.[6-10] Otten[11, 12] dehydrated a specially prepared vaccine which was reported to have remained stable in viral infectivity for many months when stored at room temperature in the tropics. This method, however, did not eliminate the many thousands of contaminants present in the virus pulp. This feature is immediately objectionable because smallpox vaccines for use in human vaccination are restricted to 1,000 organisms per milliliter or less by the standards set by the National Institute of Health.[13]

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Received for publication, June 18, 1953.

The objection to the contaminating organisms found in vaccine pulp led Rivers and Ward[10] to produce a bacteria-free virus propagated in tissue culture. Goodpasture and Buddingh[14] and others were able to produce bacteria-free vaccines using this method of virus propagation.

In our experiments we have used vaccine pulp because we have been able to reduce the bacterial contaminants in this product to an acceptable number simultaneously with the production of the vaccine.

EXPERIMENTAL

A. *Preparation and Dehydration of the Vaccine.*—The curetted pulp from the abdomen of successfully vaccinated calves is ground through a No. 60 mesh stainless steel sieve. A 20 per cent emulsion of the ground pulp is then prepared by suspending it in calf globulin in the proportion of one part pulp to four parts of globulin. Since the calf globulin contains concentrations of 0.6875 per cent phenol and 1 per cent ether*, the resulting concentrations in the emulsion will be 0.5 per cent and 0.8 per cent, respectively. The emulsion is again passed through the No. 60 mesh sieve. A volume of one milliliter is placed with aseptic technique, and with the aid of a syringe equipped with a long hypodermic needle, at the bottom of each dehydration container to be used.† The contents of the containers are shell frozen or pellet frozen and are put in the deep freezer overnight. The following morning they are dehydrated. Prior to the dehydration of the vaccine the lyophile apparatus is run with blank containers for at least three hours to eliminate the moisture and air that is present not only in the closed system but in the oil of the pump and in the mercury of the McLeod gauge.[15]

For the first two hours of dehydration the vessels containing the vaccine are kept in a freezing mixture at −75° C. The temperature of the mixture is then allowed to warm to room temperature. The vaccine is dehydrated for five hours longer. The degree of vacuum attained and maintained throughout the dehydration procedure should range between one and two microns of mercury. The glass containers are then sealed off using an oxygen-gas flame. Cracks in the glass or imperfect seals must be avoided because loss of vacuum means rapid deterioration of the vaccine.

B. *Testing the Potency of the Vaccine on the Skin of the Rabbit*[13].—A sealed container is chosen from the lot to be tested, and the outside surface is thoroughly washed with 70 per cent alcohol. A scratch is made in the neck of the container and it is opened by pressure at the scratch between several layers of sterile gauze. It is immediately plugged with sterile nonabsorbent cotton. To the dehydrated mass is added 0.66 ml. of cold sterile saline‡ to replace the fluid removed from the original volume. More fluid is added to the container to make the necessary first dilution. The container is replugged and agitated gently for a few minutes to suspend and homogenize the dehydrated mass in the liquid. The desired serial dilutions are then made and they are kept cold until they are used on the test animals.

Normal, clear-skinned, six to nine pound rabbits, thoroughly depilated dorsally, are used. Two-tenths milliliter of each dilution is pipetted on a designated portion of the back of the rabbit, and the skin under the fluid is gently but firmly irritated with a sterile needle with long parallel strokes. The area covered must be approximately 2.5 by 5 cm.

Each dilution is tested simultaneously on each of two rabbits. To determine the virus susceptibility of the animals, controls are done on the same rabbits using known potent vaccine. The animals are observed for six to seven days. They are examined daily for degree of reaction from the third day to the last. The results obtained in the two rabbits must conform very closely, otherwise new tests are performed.

*Attention footnote to Table II on preparation of calf globulin.

†The glass containers are made from Pyrex glass and are shaped like a flask. They are large enough to contain approximately 12 ml. of fluid.

‡Cold 50 per cent glycerol or distilled water may be used without altering the results.

RESULTS AND DISCUSSION

We have used, in our experiments, virus propagated in calf lymph because the supply from this source is easily available to us and abundant. Our efforts, then, were directed toward finding a means of diminishing the number of contaminants found in this product without causing significant deterioration of the potency of the vaccine. The use of antibiotics was ruled out because their presence in a vaccine that was to be used for human vaccination might cause fears of sensitization or of causing untoward reactions in people already sensitive to these substances. Satisfactory results were obtained (Table I) when the vaccines were prepared in calf globulin which contained enough phenol and ether to cause the final emulsion of lymph to have concentrations of 0.5 per cent phenol and 0.8 per cent ether.

It is to be emphasized at this point that during the period of virus propagation the calves are sprayed twice daily with various dilutions of "Roccal."* The amount of this reagent that remains in the lymph and its possible aid to the action of phenol and ether used in reducing the bacterial count is being investigated in our laboratory currently.

TABLE I. THE REDUCTION OF THE BACTERIAL COUNT OF 20 PER CENT SMALLPOX VACCINE EMULSION TO AN ACCEPTABLE NUMBER* WHEN IT IS PREPARED IN CALF GLOBULIN AND IT CONTAINS 0.5 PER CENT PHENOL AND 0.8 PER CENT EITHER

PREP. NO.	STATE OF DRYNESS OF THE VACCINE	STORAGE		BACTERIAL COUNT/ML.	
		TEMPERATURE (C.)	NO. OF DAYS	VACCINE CONTAINING 0.5% PHENOL AND 0.8% ETHER	VACCINE CONTAINING NO BACTERICIDAL AGENT
13	Not dehydrated†	—	0	—	200,000
13	Dehydrated	Minus 28°	517	50	
13	Dehydrated	Minus 28°	487	0	
		20°-25°	30		
13	Dehydrated	20°-25°	517	150	
13	Dehydrated	Minus 28°	487	175	
		37.5°	104		
13	Dehydrated	20°-25°	591	325	
13	Not dehydrated†	Minus 28°	591	—	160,000
18	Not dehydrated†	—	0	—	160,000
18	Not dehydrated‡	Minus 28°	232	100	
18	Dehydrated	Minus 28°	232	500	
18	Dehydrated	20°-25°	232	250	
18	Not dehydrated‡	Minus 28°	423	100	
18	Dehydrated	Minus 28°	423	150	
18	Dehydrated	20°-25°	423	250	
18	Not dehydrated†	Minus 28°	423	—	150,000
19	Not dehydrated†		0	—	190,000
19	Not dehydrated‡	Minus 28°	43	75	
19	Dehydrated	20°-25°	43	75	
19	Dehydrated	20°-25°	26	25	
		37.5°	18		
19	Not dehydrated‡	Minus 28°	212	300	
19	Dehydrated	20°-25°	212	450	
19	Not dehydrated†	Minus 28°	212	—	180,000

*The National Institute of Health restricts the vaccine to 1,000 organisms per milliliter or less.

†Bacterial count control of vaccine not dehydrated and prepared without phenol and ether.

‡Bacterial count control of vaccine not dehydrated but prepared with 0.5 per cent phenol and 0.8 per cent ether.

*Roccal—a germicide sold by the Winthrop Chemical Co., Windsor, Canada.

It is evident from the data submitted in Table III and others that the potency of our vaccines was not reduced perceptibly by the action of the concentrations of phenol and ether used.

Calf globulin was chosen as the suspensoid in the preparation of the vaccines because it contains per unit of volume concentrations of the euglobulin and pseudoglobulin fractions of serum proteins and any other possible factor that could account for the good virus protection results obtained by Goodpasture and Buddingh[2] and Buddingh[3, 4] when they used rabbit and beef serums as protective colloids in the preparation of their smallpox vaccines.

The test for viral potency as performed on the skin of the rabbit is after the method of Force and Leake,[16] following more strictly the modifications in technique and interpretation of results as required by the National Institute of Health.[13] According to these specifications a smallpox vaccine to be accept-

TABLE II. COMPARISON OF THE STABILITY OF THE POTENCY OF SMALLPOX VACCINE WHEN PREPARED AND DEHYDRATED IN A BASE OF GUM ACACIA, CALF GLOBULIN AND HUMAN PLASMA AND STORED AT 20° TO 25° C.

PREP. NO.	NO. OF DAYS OF STORAGE AT 20°-25° C.	DILUENT USED IN PREPARING THE 20% EMULSION OF VIRUS VACCINE	INFECTIVITY FOR THE RABBIT: % "TAKES"* DILUTIONS OF DEHYDRATED VIRUS VACCINE MADE IN SALINE						
			1/M†	1/3M	1/10M	1/20M	1/30M	1/40M	1/80M
7	44	20% Gum acacia‡	10/100§	5/100	0/100	0/90	0/90	0/85	0/50
8	48	20% Gum acacia‡	50/100	30/100	0/100	0/90	0/80	0/70	0/50
9	46	30% Gum acacia‡	10/100	5/100	0/100	0/100	0/100	0/100	0/50
10	50	Calf globulin‖	100/100	100/100	100/100	90/100	85/100	80/100	50/95
16	63	Calf globulin‖	100/100	100/100	100/100	100/100	100/100	100/100	100/100
19	43	Calf globulin‖	100/100	100/100	100/100	100/100	100/100	95/100	40/100
3	133	Human plasma	100	100	100	50	40	0	0
3	146	Calf globulin	100	100	100	50	40	0	0
Control not dehydrated	0	Saline	100	100	100	70	30	0	0

*The per cent value is an estimation of the portion of vaccinated area that shows confluent lesion. The minimal report of 5 per cent means at least one or two pustules in the area.

†M = 1,000.

‡The gum acacia solutions were prepared in saline and sterilized in the autoclave.

§Numerator = infectivity after dehydration and indicated storage period.

Denominator = infectivity after dehydration with no storage period.

‖The calf globulin was prepared by precipitating heat inactivated calf serum with 50 per cent saturation of ammonium sulfate. The precipitate was collected by filtration and dialyzed against sterile distilled water until free of sulphate ion. 1 per cent NaCl, 0.6875 per cent phenol, and 1.0 per cent ether were added and the product was sterilized by Berkefeld filtration.

The total solids content of this product was 18 per cent.

able for human vaccination must cause in rabbits of good susceptibility lesions amounting to:

> "80 to 100 per cent confluency in the 1:M* dilution: no more than a 20 per cent reduction in the 1:3M dilution: at least one discrete lesion in the 1:30M dilution, and with an approximately proportionate number of discrete lesions in the 1:10M dilution."

It is evident from the data in the tables submitted that the dilutions employed in our tests went a great deal beyond these requirements. The purpose, of course, was to present a more complete picture of the durability of the dehydrated virus preparations.

The protection afforded smallpox virus by calf globulin was found to be much greater than that of 20 per cent and 30 per cent gum acacia (Table II). There are indications, however, that human plasma renders approximately the same protective action as calf globulin (Table II).

Smallpox vaccines prepared and dehydrated as described show little deterioration of viral infectivity when stored for thirty days at 20° to 25° C., (Table III). Moreover, they seem durable enough to be suitable for human vaccination by the National Institute of Health standards[13] for five hundred and ninety-one days, or possibly longer, when exposed to a temperature of 20° to 25° C. (Table III) and for at least thirty days when exposed to a temperature of 37.5° C. (Table IV). Indeed, one preparation was still suitable for human vaccination after one hundred eighty-six days at the latter temperature (Table IV).

It became of interest at this point in our experiments to determine the period of time that our dehydrated vaccines could be stored at –28° C. as re-

TABLE III. THE STABILITY OF THE POTENCY OF SMALLPOX VACCINE WHEN PREPARED IN CALF GLOBULIN, DEHYDRATED AND STORED AT 20°-25° C. FOR 30 DAYS AND OVER

PREP. NO.	PERIOD OF STORAGE: NO. OF DAYS AT 20° TO 25° C.	INFECTIVITY FOR THE RABBIT: % "TAKES" DILUTIONS OF DEHYDRATED VACCINE MADE IN SALINE							
		1/100	1/M	1/3M	1/10M	1/20M	1/30M	1/40M	1/80M
Control Vac. not Dehydr. (ea. Prep.)	0	—	100	100	100	100	100	100	100
10	30	—	100	100	100	100	90	90	90
16	30	—	100	100	100	100	90	90	80
18	31	—	100	100	100	100	90	90	80
19	30	—	100	100	100	100	100	100	80
18	101	—	100	100	100	100	100	90	60
16	142	—	100	100	100	80	80	80	80
10	207	—	100	100	100	100	80	40	20
19	212	—	100	70	30	25	20	5	0
20	213	—	100	80	60	15	10	5	0
18	232	—	100	90	80	60	60	60	60
16	312	90	80	70	40	40	30	30	0
13	337	100	100	90	80	30	20	20	5
18	423	100	100	80	40	30	20	20	5
13	591	100	95	70	30	20	10	0	0

After the period of storage and temperature indicated, the above preparations were still suitable for human vaccination by National Institute of Health standards of infectivity.[13]

*Throughout this publication M means 1,000.

serve stock. Emergencies, then, could be met by these reserves if the stored vaccines could withstand well subsequent high temperatures for the minimal period of thirty days. Obviously, this treatment of the vaccine would simulate routine cold storage and conditions usually associated with the subsequent transportation and its use in the field in warm climates without refrigeration.

The dehydrated vaccines used for this phase of the work showed almost insignificant deterioration effect when tested on the rabbit in dilution up to 1/80M after a period of four-hundred and eighty-seven days of storage at –28° C. They showed no deterioration effect at all when tested to the same dilution after four hundred and twenty-three days of storage at –28° C. Subsequently, when these vaccines were exposed to temperatures of 20° to 25° C. and 37.5° C. for thirty days, their viral potency was more than sufficiently high and well able to conform with National Institute of Health standards of infectivity for vaccines to be used for human immunization (Table IV).

TABLE IV. THE STABILITY OF THE POTENCY OF DEHYDRATED SMALLPOX VACCINE PREPARED IN CALF GLOBULIN WHEN EXPOSED TO A TEMPERATURE OF 37.5° C. AND TO COMBINATIONS OF TEMPERATURES OF 37.5° C., 20°-25° C., AND MINUS 28° C.

PREP. NO.	PERIOD OF STORAGE: NO. OF DAYS	PERIOD OF STORAGE: TEMPERATURE C.	INFECTIVITY FOR THE RABBIT: % "TAKES" DILUTION OF DEHYDRATED VACCINE MADE IN SALINE: 1/100	1/M	1/3M	1/10M	1/20M	1/30M	1/40M	1/80M
19*	30	37.5°	100	100	100	100	90	90	70	50
20*	43	37.5°	100	100	80	20	10	10	10	5
20	56	37.5°	100	80	60	10	5	0	0	0
21*	186	37.5°	100	90	90	70	50	30	30	10
20	213	37.5°	90	30	20	20	20	10	5	0
19*	26	20°-25°								
	18	37.5°	100	100	100	100	85	50	20	20
19*	26	20°-25°								
	30	37.5°	100	100	90	90	20	20	20	5
19	26	20°-25°								
	93	37.5°	100	80	60	10	0	0	0	0
13*	487	Minus 28°								
	30	20°-25°	100	100	100	80	50	40	40	20
13*	487	Minus 28°								
	30	37.5°	100	80	80	60	40	30	30	10
13	487	Minus 28°								
	104	37.5°	100	80	40	10	10	10	5	0

*At the end of the period of storage and temperature indicated these preparations were still suitable for human vaccination by National Institutes of Health standards of infectivity.[13]

It may be emphasized that to dehydrate successfully smallpox vaccine prepared in calf globulin it is of the utmost importance to dehydrate for the first two hours at a very low temperature as described. Attaining and maintaining a negative pressure of between one and two microns of mercury throughout the dehydration and at the sealing of the glass containers is of equal importance.

SUMMARY

Highly contaminated smallpox calf lymph was reduced in bacterial count until it became acceptable for the production of vaccines to be used for human

vaccination. This effect was obtained when the lymph we used was ground in calf globulin and the resulting emulsion contained 0.5 per cent phenol and 0.8 per cent ether.

When dehydrated as described, this type of vaccine suffered little if any loss of viral potency during the dehydration process.

The dehydrated vaccine when stored at −28° C. for more than a year suffered little appreciable loss of viral potency.

The vaccines that were stored for more than a year at 20° to 25° C. and for thirty to forty days, and in one instance for one-hundred and eighty-six days, at 37.5° C. were still potent enough to warrant their use for human vaccination at the end of these periods of time.

The viral potency of the vaccine preparations we have described was determined and interpreted according to the standard method as performed on the skin of the rabbit and as required by the National Institute of Health.

The author acknowledges the assistance rendered by Anthony Pignataro and Gloria Mamber Schultz in the performance of the many animal tests.

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TOCOPHEROL RESPONSE CURVES AND FAT ABSORPTION

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IMPORTANT relationships between altered serum tocopherol levels and physiologic inadequacy have not been satisfactorily demonstrated in man. However, studies indicate a less than normal plasma tocopherol level in malnourished individuals, and in disease states associated with impaired absorption of fat. High serum tocopherol levels have been found in conditions associated with hypercholesteremia, although no quantitative relationship has been demonstrated.[1] Abnormally flat tolerance curves following the oral administration of tocopherol have been reported in some cases of cirrhosis of the liver.[2] Experimental chronic biliary fistula in dogs on a normal diet, causing diversion of bile from the intestinal tract, produces typical muscular dystrophy and testicular atrophy of vitamin E deficiency.[3] Presumably, these effects and the poor response curves are due to faulty absorption of the vitamin. The following studies were planned to determine, in normal individuals, whether tocopherol levels are altered by low fat intake; whether absorption of vitamin E as determined from tolerance curves can be altered by simultaneous feeding of vitamin E and a fatty meal; and whether absorption of vitamin E can be increased by oral administration in aqueous dispersion.

MATERIALS AND METHODS

Two groups of subjects were studied: (a) Twelve normal adults ranging in age from 22 to 55 years, free of any history of liver disease or of any recent illness. (b) Three adults who had been on a low fat, low cholesterol diet for a period of time long enough to achieve significant lowering of plasma lipid levels.

Serum tocopherol concentrations were determined by a macromodification of the Quaife Biehler method.[4, 5]

Tocopherol tolerance tests were carried out as follows. After a fasting specimen was drawn in the early morning, 1,500 mg. of tocopherol was administered orally and blood samples were drawn three, six, nine, twelve, twenty-four, and forty-eight hours later. The preparation of vitamin E in oil was E-Toplex.* Each capsule provides d-alpha tocopheryl acetate equivalent in vitamin E biological activity to 100 mg. alpha tocopherol. The vitamin E in aqueous dispersion was Aquason-E.* Each capsule contains 100 mg. of equal biological activity. Two weeks were allowed to lapse between each test period.

In the first test period, subjects were kept on a fat-free diet during the experimental procedures. Later these tests were repeated, allowing a heavy fat breakfast containing at least 5 ounces of fat in addition to 1,500 mg. of a tocopherol preparation.

Fluctuations of serum tocopherol which might be spontaneous or due to diet were measured by drawing blood at intervals for a twenty-four hour period in 4 of the normal subjects prior to the absorption tests.

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Received for publication, July 3, 1953.

*Kindly provided by Dr. Louis Freedman, Research Director of the U. S. Vitamin Corporation.

RESULTS

Table I demonstrates the concentrations of plasma tocopherol obtained in 12 normal individuals before and after a 1,500 mg. dose of vitamin E in oil and in aqueous dispersion. All subjects were kept on a fat-free diet during the investigative period. Fig. 1 demonstrates maximal, minimal, and average plasma tocopherol concentrations. Fasting serum concentrations range from 0.35 mg. per cent to 1.48 mg. per cent. The maximum rise achieved with the aqueous dispersion was significantly greater than that following the oil preparation, and maximum elevation was achieved in a shorter time in the majority of subjects tested. The most marked absorption differences between tocopherol in oil and that in an aqueous media were seen in those who showed little elevation following tocopherol in oil. It was in these cases that aqueous dispersion of vitamin E achieved a significantly increased absorption.

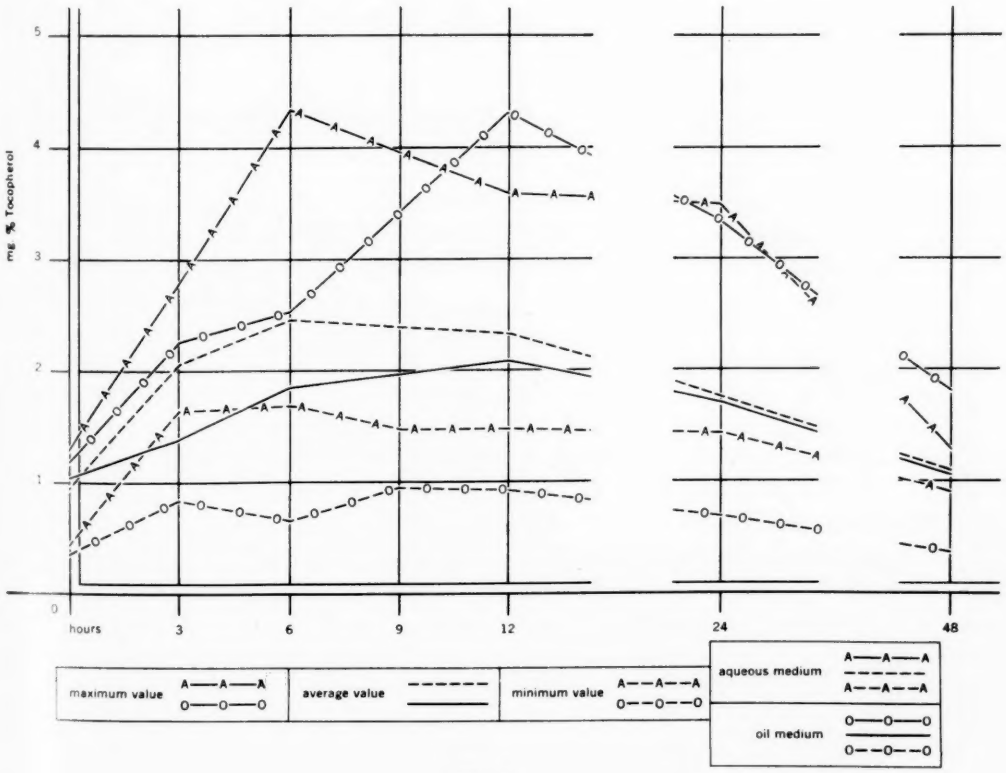

Fig. 1.

In Table II is demonstrated the daily variation in plasma tocopherol levels under controlled dietary conditions. No significant changes are noted.

Table III shows the effect of feeding a high fat meal together with 1,500 mg. of tocopherol in oil. Those subjects with a previously flat tolerance curve showed no additional response to the added fatty meals. Subjects in whom

TABLE I. COMPARISON OF SERUM TOCOPHEROL LEVELS AFTER ADMINISTRATION OF 1,500 MG. NATURAL D,ALPHA-TOCOPHERYL ACETATE IN OIL AND AQUEOUS MEDIUMS

PATIENT		HOURS 0	3	6	9	12	24	36	48	MAX. RISE	HOURS
M. R.	oil	0.35	0.84	0.68	0.94	0.92	0.62		0.38	0.49	9
	aqueous	0.44	1.62	1.68	1.42	1.42	1.40	1.24	0.90	1.24	6
M. S.	oil	0.80	1.28	2.16		2.40	1.32		1.24	1.60	12
	aqueous	0.94	1.86	2.48		2.44	1.26		1.12	1.54	6
J. R.	oil	0.80	1.60	2.24		2.48	2.32		1.02	1.68	12
	aqueous	0.70	1.91	2.23	2.23	2.34	2.30		1.62	1.64	12
S. H.	oil	0.92	1.45	1.30		1.12			0.96	0.53	3
	aqueous	0.70	1.80	1.70		1.70		1.68	0.77	1.10	3
S. F.	oil	1.07	1.31	2.18		2.38	1.83		1.20	1.21	12
	aqueous	1.03	2.42	3.0		3.24	1.36		1.32	2.21	12
Y. C.	oil	1.22	2.28	2.50		4.30	3.34		1.28	3.08	12
	aqueous	1.28	2.76	4.39		3.60	3.50		1.20	3.11	6
G. K.	oil	1.15	1.23	2.0	2.6	2.1	2.60		1.25	1.45	24
	aqueous	1.26	2.10	2.85		2.84	2.50	1.24		1.59	6
R. G.	oil	1.12	1.32	1.52		1.41	1.12		1.02	0.40	6
	aqueous	0.92	1.46	1.40		1.30	1.14		0.98	0.54	3
B. S.	oil	1.10	1.20	1.23		1.24	1.20		1.0	0.14	12
	aqueous	1.0	1.96	1.73	1.80	1.80	1.43		1.2	0.96	3
B. B.	oil	1.28	1.28	1.36		1.52	1.48		1.32	0.24	12
	aqueous	1.34	2.11	2.36		2.02	1.26		1.30	1.02	6
A. S.	oil	1.24	1.32	1.46		2.0	1.84		1.40	0.76	12
	aqueous	1.20	1.43	2.84		2.80	1.42		1.26	1.64	6
I. P.	oil	1.48	1.47	2.96		3.14	1.42		1.53	1.66	12
	aqueous	1.36	3.32	3.12		3.02	1.68		1.44	1.76	3

vitamin E absorption was good gave increased tocopherol levels following simultaneous feeding of a fatty meal. A 2 to 3 plus lipemia was noted in the six hour specimen in these subjects. Y. C. (Table I), who achieved the highest

TABLE II. SERUM TOCOPHEROL LEVELS IN RELATION TO NORMAL DIET WITHOUT ADDED TOCOPHEROL

PATIENT	9 A.M.	12 NOON	4 P.M.	9 A.M.
M. R.	0.35	0.43	0.58	0.45
S. H.	0.94	1.02	1.08	0.78
C. K.	1.14	1.18	1.22	1.06
I. P.	1.48	1.48	1.46	1.36

tocopherol levels, demonstrated a 2 plus serum lipemia in the twelve-hour specimen, although no fat was given during the test period.

Table IV includes tocopherol tolerances in individuals maintained on low fat intake for at least six weeks prior to and during the test period. In all

TABLE III. SERUM TOCOPHEROL LEVELS AFTER ORAL ADMINISTRATION OF 1,500 MG. NATURAL D,ALPHA-TOCOPHERYL IN OIL WITH AND WITHOUT FATTY BREAKFAST

		0	3	6	9	12	24	36	48
M. R.	No breakfast	0.35	0.84	0.68	0.94	0.92	0.62		0.38
	Fatty breakfast	0.38	0.82	0.74	0.84	0.86	0.78		0.44
J. R.	No breakfast	0.80	1.60	2.24		2.48	2.32		1.02
	Fatty breakfast	0.72	1.62	3.44 (2+ lipemia)		3.24	2.16		0.96
B. B.	No breakfast	1.28	1.28	1.36		1.52	1.48		1.32
	Fatty breakfast	1.30	1.36	1.44		1.60	1.62		1.14
I. P.	No breakfast	1.48	1.47	2.96		3.14	1.42		1.53
	Fatty breakfast	1.41	1.56	3.95 (2+ lipemia)		4.10	2.01		1.46

TABLE IV. SUBJECTS PREVIOUSLY MAINTAINED ON EXTREMELY LOW FAT INTAKE FOR AT LEAST SIX WEEKS. TOCOPHEROL TOLERANCE WITH 1,500 MG. VITAMIN E

		0	3	6	9	12	24	48
S. R. B.	Vitamin E in oil	0.43	0.38	0.58	0.69	0.60	0.64	0.62
	Vitamin E in aqueous medium	0.49	0.94	1.24		1.20	0.68	0.53
	Vitamin E in oil and fatty meal	0.52	0.56	0.88		0.92	0.58	0.60
M. P.	Vitamin E in oil	0.79	0.74	0.84		0.98	0.82	0.73
	Vitamin E in aqueous medium	0.73	1.25	1.48		1.54	0.94	0.78
	Vitamin E in oil and fatty meal	0.78	0.74	1.02		1.04	0.88	0.88
G. K.	Vitamin E in oil	0.38	0.68	0.54		0.70	0.44	0.42
	Vitamin E in aqueous medium	0.36	0.93	1.10		1.02	0.88	0.56
	Vitamin E in oil and fatty meal	0.54	0.65	0.63		0.72	0.72	0.74
	Vitamin E in aqueous and fatty meal	0.46	0.88	1.35		1.30	1.10	1.02

subjects, fasting tocopherol levels were low and a test dose of 1,500 mg. vitamin E in oil produced a minimal response. Levels following a similar tocopherol dose in aqueous dispersion were significantly elevated. The inclusion of a fatty meal with the test dose in oil elicited a significantly increased response in these subjects. In one subject in whom the aqueous dispersion preparation was administered with a fatty meal, surprisingly higher levels were achieved than with any other combination.

DISCUSSION

The absence of an expected elevation of serum tocopherol concentration after oral vitamin E administration may be attributed either to impaired intestinal absorption or to an increased removal rate from the blood. Previous studies[6] indicate the response curve to be a reasonable reflection of efficiency of absorption which is little influenced by the state of tissue storage or utilization of tocopherol. The present results, indicating a greater response when vitamin E in aqueous dispersion was given as the test substance, tend to substantiate these findings. There also is no apparent relationship between the fasting serum tocopherol level and the anticipated response curve.

Vitamin E is apparently absorbed from the intestinal tract by the same mechanism as exogenous fat, or together with it. The process of intestinal fat absorption is inhibited following a period of low fat intake, but is quickly reactivated by a fatty meal. In those subjects not responding to a fatty meal, the fat absorptive ability of the intestinal tract may be seriously impaired. It is in this type of disturbance that the aqueous dispersion of oil soluble vitamins is most important. This disturbance may involve intestinal hormones associated with gall bladder function and the excretion of bile. The effect of bile secretion on vitamin E absorption is being studied further. The particle size in the aqueous dispersion may obviate the necessity for emulsification by biliary secretion.

Under normal physiologic conditions the absorption of vitamin E from the intestinal tract is good and does not require a dispersing agent. In those conditions of impaired absorption of fat and fat soluble vitamins, vitamin E in an aqueous dispersion may play an important nutritional role. Prolonged fat

restriction in an attempt to lower serum cholesterol levels in atherosclerosis may lead to a significant vitamin E deficiency unless a supplementary aqueous dispersion of vitamin E is administered.

The presented data suggest the comparison response of vitamin E in oil and that in an aqueous dispersion as an excellent test of fat absorption.

SUMMARY AND CONCLUSIONS

The range and distribution of serum tocopherol values is extremely wide in a group of normal individuals. There is a significant absorption variability in these normal subjects. Intestinal absorption of fat and fat soluble vitamins appears to be related to antecedent dietary fat intake. Tocopherol in aqueous dispersion permits greater tocopherol absorption in those subjects demonstrating impaired absorption of tocopherol in an oily medium. The degree of intestinal absorption of tocopherol cannot be predicted from the fasting plasma tocopherol level.

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INFLUENCE OF ALCOHOL CONSUMPTION ON HEPATIC FUNCTION IN HEALTHY GAINFULLY EMPLOYED MEN

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THE occurrence of liver disease and function test abnormalities following persistent excessive alcohol consumption is well known and commonly seen in general hospitals. The liver disease is presumed to follow nutritional deficiencies which commonly accompany chronic alcoholism. However, objective information concerning liver function changes in healthy individuals with variable alcohol intake is lacking. The availability of a large group of gainfully employed men, called in as part of a follow-up survey, and of semiquantitative estimates of alcohol intake enabled us to study this problem.

METHODS AND MATERIALS

Case Material.—Data are available on 744 presumably healthy men who were called in for examination during a survey of veterans.[1] Approximately one-half had had acute viral hepatitis four to six years prior to examination. The remainder had never had any diseases of the liver. The proportions of cases consuming various amounts of alcohol were similar for each group. Physical examination was essentially normal in each instance. No attempt was made to eliminate cases because of incidental factors such as occupational exposures of possible but unknown significance. Dietary habits were satisfactory, even in the case of those consuming large amounts of alcohol, and there were no evidences of deficiencies.

Alcohol Consumption.—Information was obtained with a ten-point rating scale (Table I) designed to provide a semiquantitative estimate of alcohol ingestion irrespective of the beverage consumed. The ten-point rating is recorded separately for each of the three major groups of beverages. For each beverage a given rating represents approximately equivalent amounts of alcohol. A single final rating is obtained by adding up quantities ingested in terms of equivalent amounts of one of the beverages. The rating corresponding to this total intake is then recorded.

The rating scale was checked by each individual privately, but was carefully reviewed with the subject during the course of the physician's systemic review. Any inconsistencies were noted by the physician, and unreliable responses were discarded.

Liver Function Tests.—Eleven tests were performed on each individual. Nine of the tests were performed simultaneously; two were performed on a twenty-four hour urine specimen collected within one or two weeks of the examination in most instances. The following tests were included:

Serum bilirubin, one-minute (1'B) and total (TB), mg. %
Thymol turbidity (TT), units
Zinc sulfate turbidity (ZT), units
Cephalin cholesterol flocculation (CCF)
Colloidal red flocculation (CR)
Per cent cholesterol esters (%CE)
Bromsulfalein retention (BSP), %

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Supported by a separate grant from the Veterans Administration upon recommendation of the Committee on Veterans Medical Problems of the National Research Council.

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication, July 7, 1953.

Intravenous hippuric acid (HA), Gm.
Intravenous galactose tolerance (GT), mg. %
Urine urobilinogen (UU), mg./day
Urine coproporphyrin (UCP), μg/day

TABLE I. ALCOHOL RATING SCALE

Check (X) the line in each group below that best describes how much liquor, wine, or beer you consume *on the average.*

Liquors (whiskey, brandy, gin, rum, etc.)	
1. 1 fifth or quart each day	
2. 1 pint each day	
3. ½ pint each day	
4. 6 ounces (jiggers, "shots") each day or 1 fifth or quart each week	
5. 1 pint each week	
6. 1-3 ounces (jiggers, "shots") each day	
7. 4-6 ounces (jiggers, "shots") each week	
8. 1-3 ounces (jiggers, "shots") each week	
9. An occasional drink each month or a number of drinks on rare occasions	
10. Never any liquor	
Wines	
1. 4 quarts each day	
2. 2 quarts each day	
3. 1 quart each day	
4. 1 pint each day or 3 quarts each week	
5. 2 quarts each week	
6. 1-3 half-size glasses each day	
7. 1 pint each week	
8. 1-3 half-size glasses each week	
9. An occasional drink each month or a number of drinks on rare occasions	
10. Never any wine	
Beer, Cider, Ale	
1. 24 bottles each day	
2. 16 glasses or 12 bottles each day	
3. 8 glasses or 6 bottles each day	
4. 4 glasses or 3 bottles each day or 18 bottles each week	
5. 16 glasses or 12 bottles each week	
6. 1-3 glasses or a bottle or two each day	
7. 4-6 glasses or 3 bottles each week	
8. 1-3 glasses or a bottle or two each week	
9. An occasional drink each month or a number of drinks on rare occasions	
10. Never any beer, cider, or ale	
Rating	

Corresponding numbers under each of the three beverage groupings represent approximately equivalent amounts of alcohol. A single final rating is obtained by adding up quantities ingested in terms of equivalent amounts of one of the beverages.

In addition a derived weighted linear combination of the tests giving the best separation between a group of normal subjects and a group of cirrhosis patients was calculated for each subject. This combination is called the cirrhosis abnormality score (CAS).[2]

Details concerning these laboratory procedures are to be found in references three and four.

Analysis of the Data.—For purposes of the analysis the ten ratings were reduced to four alcohol rating (AR) groups as follows:

Rating Group	*Consumption equal to*
AR 1-2	One pint or more of liquor each day
AR 3-4	One fifth each week to one-half pint each day
AR 5-8	One ounce to one pint each week
AR 9-10	None, an occasional drink per month, or a number of drinks on rare occasions

Frequency distributions were plotted for each group and each test, and means ($\bar{X}$), standard deviations (S), and percentages of cases exceeding certain limits of each test were calculated. Where appropriate, tests of significance of differences between means and between percentages were calculated utilizing critical ratios (difference divided by its standard error) and chi square, respectively. In those instances where a trend was evident, though the individual differences between means were not significant, probabilities from the significance tests were combined appropriately[5] and the significance of the combination evaluated by means of a chi square test.

Certain descriptive characteristics—age, weight, and surface area—of each rating group are summarized in Fig. 1.

RESULTS AND INTERPRETATIONS

The quantitative tests are summarized in Figs. 2, *A* and 2, *B*. Group AR 1-2 consisted of only fourteen subjects, so marked irregularity of its frequency distribution curve is to be expected.

The various group means for the one-minute bilirubin are insignificantly different. The mean total bilirubin for Group AR 1-2 was 0.47 mg. per cent. This is probably a significantly lower value than the means of the other groups (critical ratio for comparison with AR 9-10 is 2.22). Those consuming large amounts of alcohol thus tend to have total bilirubin values that are "more normal" than those of the more moderate drinkers.

The various group results were essentially similar for the thymol turbidity test. The means for the zinc sulfate turbidity test show a suggestive sequential increase with decreased consumption, however, none of the individual mean differences was significant, nor was a combination of the probabilities resulting from the tests of significance.

The suggestive decrease in total cholesterol with decrease in consumption is adequately accounted for by the age differences between the groups and the known relationship of cholesterol to age.[6]

The per cent cholesterol esters shows a definite trend with reduction of the mean values as consumption increases. None of the comparisons among means is significant, though comparison of AR 1-2 and AR 9-10 yielded a critical ratio of 1.94. A combination of the probabilities resulting from the three tests of significance of differences between the means of adjacent groups yields a significant value (χ^2 for 6 degrees of freedom $= 13.5$, $p < 0.05$). The trend is thus suggestive of a real association with alcohol consumption.

The intravenous galactose tolerance, urine urobilinogen, and bromsulfalein tests do not show any group differences of note. Neither does the cirrhosis abnormality score.

A finding similar to that for the total bilirubin is noted with the intravenous hippuric acid test. The value for AR 1-2 is "more normal" than those for the other groups (critical ratio for comparison of AR 1-2 and AR 9-10 is 2.03). The same trend is noted with the bromsulfalein test, though the difference is not as marked.

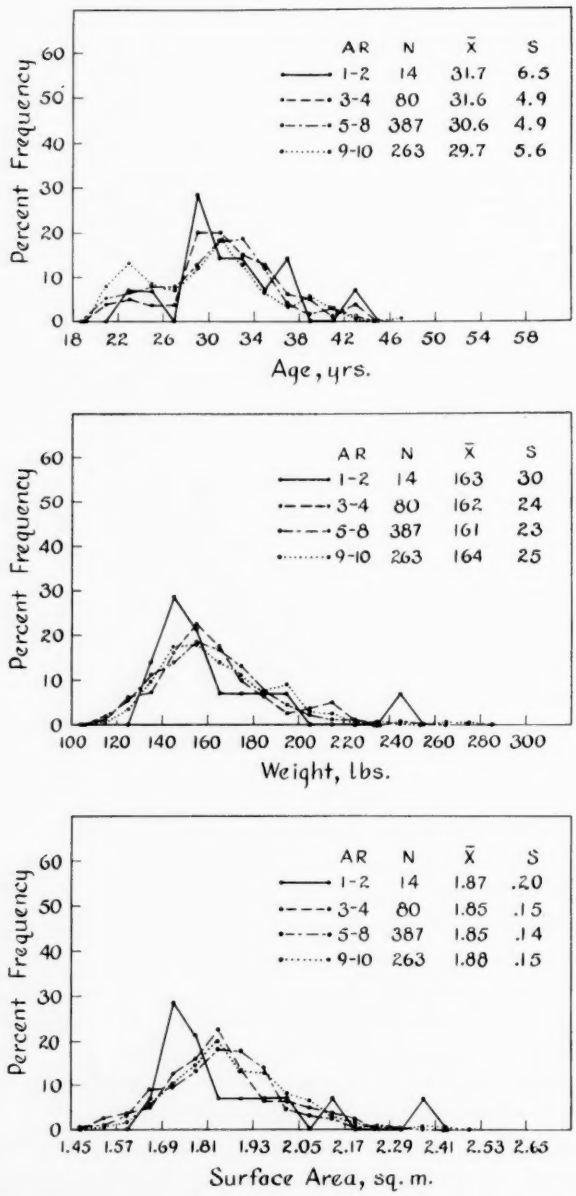

Fig. 1.—Descriptive characteristics of alcohol rating (AR) groups.

The urine coproporphyrin test is the only one showing an unequivocal relationship to alcohol consumption. The greater the consumption the more coproporphyrin excreted. Comparison of mean values for AR 1-2 and AR 9-10 yielded a critical ratio of 3.95; for AR 3-4 and AR 9-10, a critical ratio of 3.33; and for AR 5-8 and AR 9-10, a critical ratio of 3.72. Comparison of the percentage of cases exceeding 300 μg. per day (Table II) shows a significant difference in the same direction. Eight and two-tenths per cent of the AR 5-8 cases compared with 3.5 per cent of the AR 9-10 cases exceeded 300 μg. per day ($\chi^2 = 5.69$, $p < 0.02$). The implications of these results have been described elsewhere.[7] This association with alcohol consumption is probably unrelated to hepatic status per se. Only one other difference in Table II is significant (comparison of AR 5-8 and AR 9-10 for bromsulfalein scores exceeding 5 per cent), and that is adequately accounted for by the relationship of the test to body weight.[3]

TABLE II. PERCENTAGE OF CASES EXCEEDING CERTAIN LIMITS OF EACH TEST

	AR 1-2			AR 3-4			AR 5-8			AR 9-10		
	N_1	N_2	%	N_1	N_2	%	N_1	N_2	%	N_1	N_2	%
1'B >0.20 mg. %	14	0	0	80	5	6.3	387	13	3.4	262	13	5.0
TB >1.0 mg. %	14	1	7.1	80	2	2.5	387	16	4.1	263	14	5.7
>1.5	14	0	0	80	0	0	387	4	1.0	263	5	1.9
>2.0	14	0	0	80	0	0	387	3	0.8	263	1	0.4
TT ≧6.0 units	12	0	0	78	3	3.8	386	14	3.6	263	18	6.8
ZT ≧15 units	14	0	0	80	0	0	386	6	1.6	263	4	1.5
%CE ≦71%	14	0	0	80	2	2.5	387	14	3.6	263	4	1.5
BSP >5%	14	2	14.3	80	19	23.7	386	74	19.2	262	70	26.7
>10	14	0	0	80	2	2.5	386	12	3.1	262	17	6.5
>15	14	0	0	80	1	1.3	386	1	0.3	262	2	0.8
GT ≧40 mg. %	14	1	7.1	79	2	2.5	384	7	1.8	257	7	2.7
HA <0.80 Gm.	13	0	0	80	1	1.3	376	15	4.0	258	15	5.8
UU >3.5 mg./day	14	0	0	76	4	5.3	364	21	5.8	252	13	5.2
≧6.0	14	0	0	76	1	1.3	364	6	1.6	252	3	1.2
UCP ≧300 μg/day	13	1	7.7	76	6	7.9	376	31	8.2	255	9	3.5
≧400	13	0	0	76	0	0	376	5	1.3	255	1	0.4
CAS ≧0 units	13	0	0	76	2	2.6	365	5	1.4	249	7	2.8

N_1 = Number of cases in group.
N_2 = Number of cases exceeding indicated limit.

Results with the qualitative tests (cephalin cholesterol flocculation and colloidal red) are summarized in Table III. There are no significant group differences.

TABLE III. PERCENTAGE OF CASES WITH "ZERO" RATINGS ON CCF AND CR TESTS, BY AR GROUP

	AR 1-2	AR 3-4	AR 5-8	AR 9-10
CCF, 24 hr.	100	99	99	100
CCF, 48 hr.	88	88	88	84
CR, 24 hr.	94	98	94	95
CR, 48 hr.	81	84	77	76

COMMENT

It is clear from this analysis that there is no prominent relationship between alcohol consumption and hepatic function in men whose nutrition and

health are normal. Relatively large amounts of alcohol may be consumed without evident abnormality of hepatic function tests. However, as has been shown by Cates[8] and Voegtlin and associates,[9] excesses leading to hospitalization for alcoholism are associated with functional abnormalities. In these instances the consumption of alcohol is such as to interfere with proper nutrition and gainful employment. Varying numbers of these individuals, by continuing their excesses, eventually develop fatty livers and portal cirrhosis.

Of special interest are the findings that excessive alcohol consumption may result in scores on certain tests, the serum bilirubin and hippuric acid tests

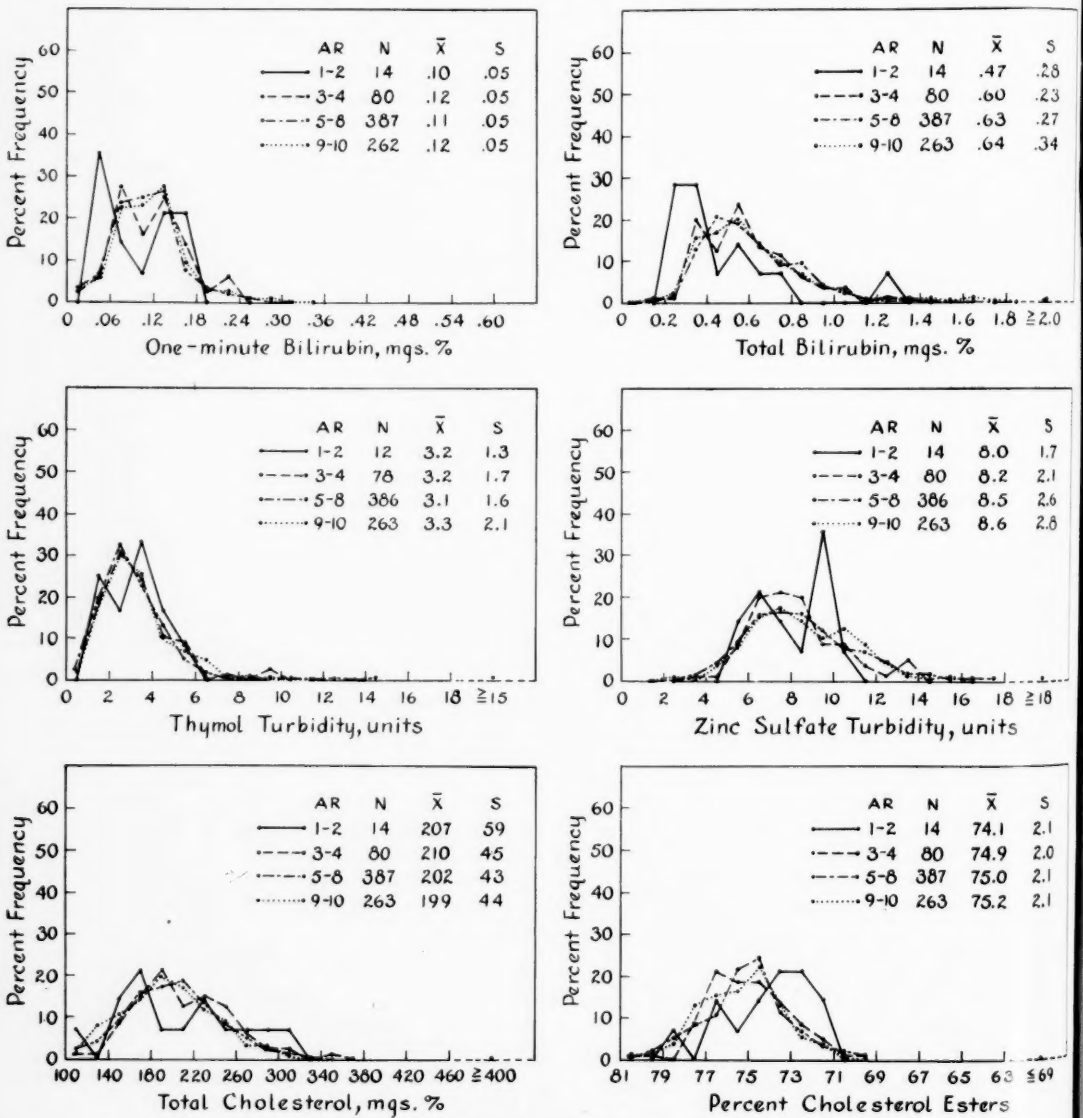

Fig. 2, *A*.—Liver function test results of alcohol rating (AR) groups.

particularly, which are "more normal" than usual. They suggest the existence of hyperfunctioning cells, possibly related to minimal damage.

Evidence of similar hyperfunctioning of hepatic cells has been presented previously. Rosenberg and Soskin[10] observed hyperexcretion of hippuric acid in thirty-nine of one-hundred patients with mild forms of liver disease. Atkinson[11] similarly observed hyperexcretion of hippuric acid in gouty patients with mild liver damage. In experimental studies Whipple and associates[12] observed increased excretion of phenoltetrachlorphthalein in a dog after chronic phos-

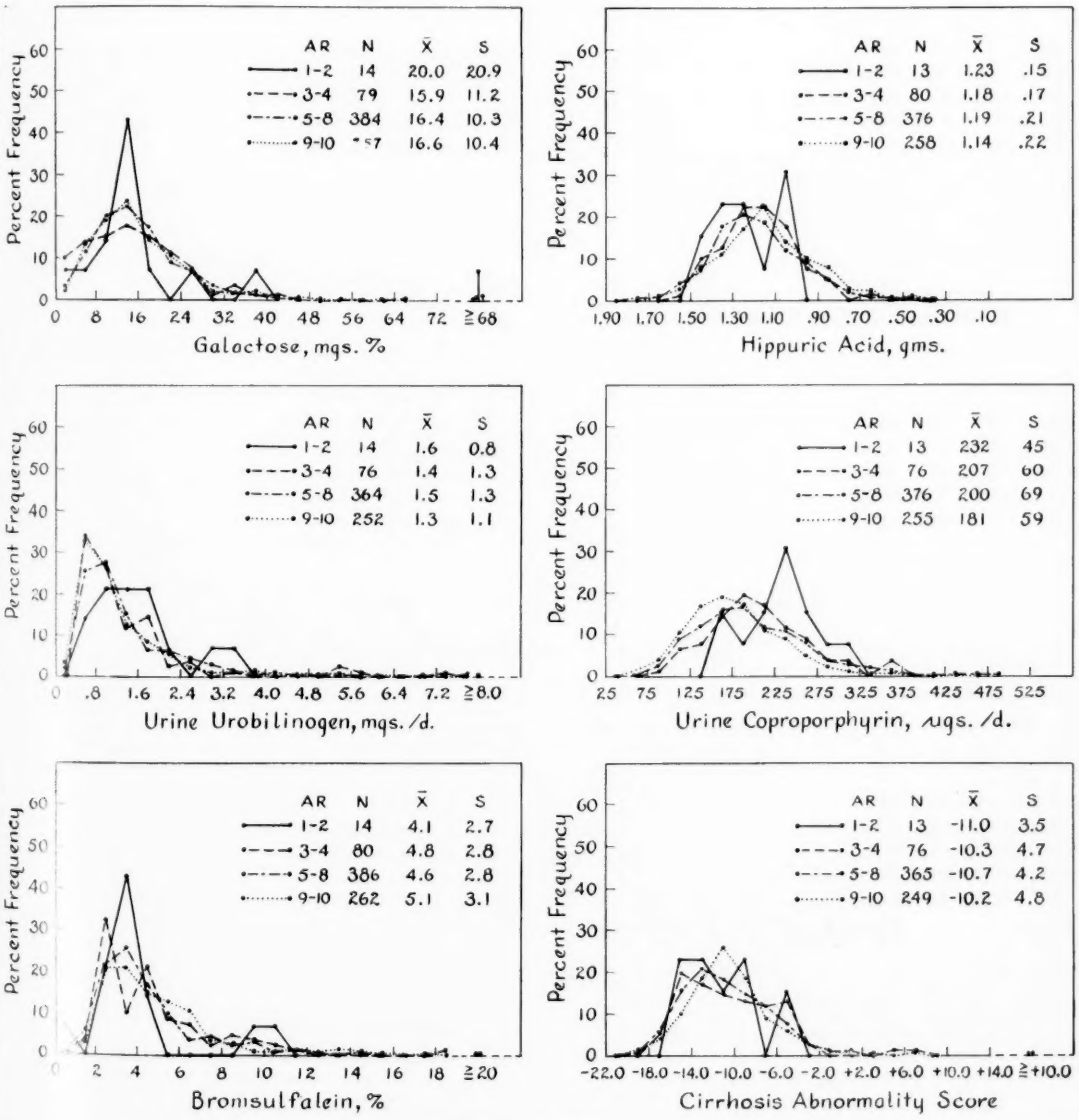

Fig. 2, *B*.—Liver function test results of alcohol rating (AR) groups.

phorous poisoning. Likewise Althausen and Thoenes[13] noticed that the epinephrine hyperglycemia of rabbits subjected to chronic phosphorous poisoning was at first excessive, later decreased, and finally abolished. These findings are analogous to the well-known pattern of the sensory nerve which responds to minimal damage with hyperesthesia and to more severe damage with hypesthesia.

SUMMARY

In general, with one exception, there is no striking relationship between alcohol consumption and liver function test results in apparently healthy gainfully employed men. Urine coproporphyrin excretion varies directly with alcohol consumption, probably not, however, as a consequence of hepatic dysfunction.

Evidence is presented that excessive alcohol ingestion may result in scores on certain tests, serum bilirubin, and hippuric acid particularly, that are "more normal" than usual. This is probably related to cellular hyperfunction associated with minimal damage.

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ANTIRABBIT PLATELET AND RED-CELL FACTORS IN NORMAL BLOOD

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INTRODUCTION

WITH the demonstration by Harrington and associates[1] of antiplatelet substances in the blood of many patients with idiopathic thrombocytopenia, the proposal that this disease may be caused by humoral agents received renewed consideration. In an effort to find a suitable animal test for further investigation, rabbits were injected with plasma and serum from a patient with idiopathic thrombocytopenia as well as from a normal control subject. A rapidly developing thrombocytopenia with death accompanied by hemorrhagic manifestations, resulted in both groups of animals. A survey of the literature prior to 1952 revealed only one reference to this phenomenon in the Japanese literature.[2] More recently it has been mentioned by Stefanini and associates.[3] This led us to a more detailed study of the effects of normal plasma and serum upon the blood picture in rabbits, which is the subject of this report.

MATERIALS AND METHODS

Rabbits were injected intravenously with varying amounts of fresh citrated human or animal plasma, fresh serum or reconstituted lyophilized pooled human plasma. Erythrocyte, total and differential leukocyte, and platelet counts were performed on ear blood of the animals prior to injection and at regular time intervals thereafter. Platelets were counted by the method of Tocantins[4] on arterial blood and with 0.5 ml. of venous blood diluted in a red-cell pipette with 3.5 per cent sodium citrate solution, containing 0.2 per cent of formalin. Counts were done in an ordinary hemocytometer chamber. Vacuum bottles of lyophilized pooled human plasma (Lyovac, Sharp & Dohme) were opened and the amount needed for an experiment was weighed out and reconstituted with a proportional amount of the supplied diluent, the remainder being stored in the refrigerator for future use.

RESULTS

Experiment I. Effect of Normal Human Plasma or Serum on Rabbit Blood Cells.—From 3 to 25 ml. of normal human serum, fresh or lyophilized plasma were injected into an ear vein of 13 rabbits. The results depicted in Tables I and II and Figs. 1 and 2 indicated a fall of platelets and red cells following the injection. This was usually apparent at one-half hour and reached a low point from one to three hours after injection. If the animals survived, the effect persisted up to forty-eight hours depending on the severity of the reaction. The effect appeared to be proportional to the amount of plasma or

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Received for publication, June 22, 1953.

TABLE I. EFFECT OF SERUM OR PLASMA ON RABBIT PLATELET LEVELS

RABBIT NO.	DOSE AND MATERIAL	PLATELET COUNTS (10^3) VENOUS BLOOD (ARTERIAL BLOOD)								
		INITIAL	½ HR.	1 HR.	2 HR.	3 HR.	4 HR.	12 HR.	24 HR.	48 HR.
1	25 ml. ITP plasma	700 (–)				x				
2	20 ml. normal plasma	550 (–)	x							
3	7 ml. ITP serum	750		170	60	90	x			
4a	3 ml. ITP plasma	900		145	160	320	370	280	150	640
4b	3 ml. normal plasma	545	450		550	500			900	
4c	5 ml. normal serum	900		80	120	146			x	
5a	5 ml. normal plasma	300		240	125	170	175		322	
5b	5 ml. normal serum	322		64	23	x				
12	10 ml. Lyovac	674 (788)	106 (60)	101 (79)		293			310 (370)	
19	20 ml. Lyovac	678 (601)	57 (20)	99		112			x	
9	10 ml. Lyovac	770	455 (479)	302 (355)		206 (250)			217 (204)	
55	10 ml. Lyovac	(326)	(178)	(242)		(155)				
57	5 ml. ITP serum	626 (680)	412 (450)	415 (380)		595				
2′	10 ml. dog plasma	518	478 (495)	429 (415)		322				
26	10 ml. dog plasma	609 (636)	592 (452)	463 (498)		500			474 (467)	
4′	10 ml. sheep plasma	542	384 (400)	365 (400)		362			363	
20	10 ml. sheep plasma	641	429 (379)	425 (415)		635			640 (591)	

ITP = Idiopathic thrombocytopenic purpura.
x = Rabbits died with evidence of bleeding.
Where the same rabbit was used for different experiments, the subscript a, b, etc., is added.
Arterial platelet counts in parentheses.

serum injected and also seemed to be greater with serum than with plasma. The white blood cell response was variable, but in most instances there was an initial lymphopenia followed in several hours by leukocytosis due to an increase of neutrophils. There was no essential difference in the changes observed in venous and arterial platelet levels.

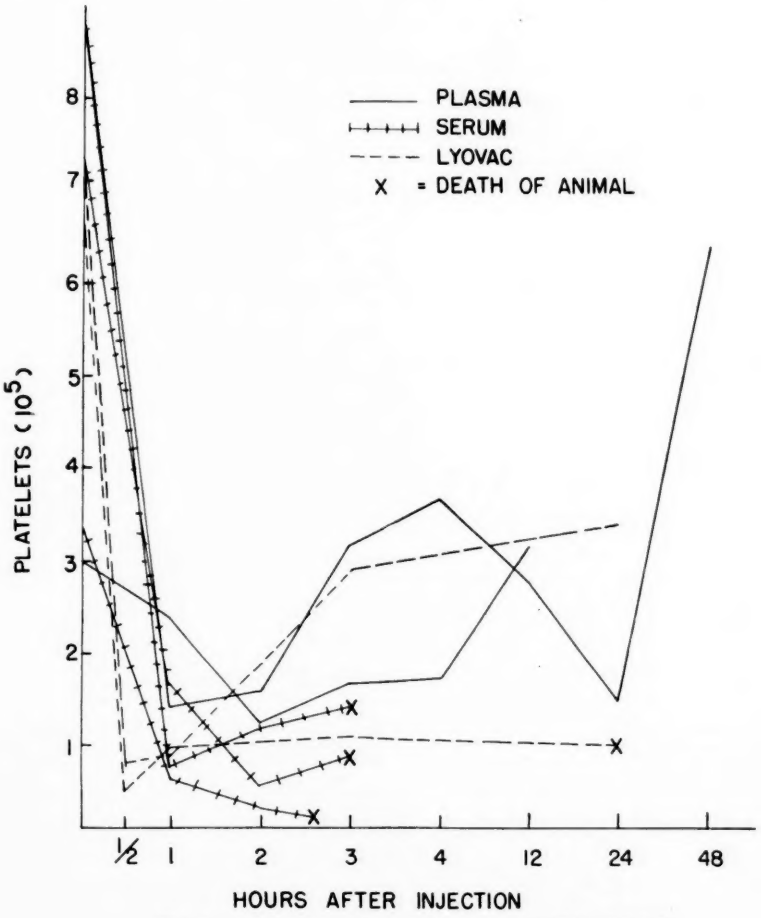

Fig. 1.—Effect of serum or plasma on rabbit platelets.

Experiment II. Demonstration of Antirabbit Cell Factors in Plasma From Other Species.—Four rabbits were injected with 10 ml. each of citrated fresh dog and sheep plasma. Anemia and thrombocytopenia again resulted (Tables I and II) but to a lesser degree than with equal amounts of fresh human plasma.

Experiment III. Effect of Heating and Aging on the Anticellular Action of Plasma.—Three rabbits were injected with 10 ml. of freshly reconstituted lyophilized human plasma which had been heated for thirty minutes at 56° C. This treatment resulted in precipitation of a heavy flocculum. The decanted supernatant was used for one injection. Samples from the same source of

Lyovac stored in the icebox were reconstituted four and fourteen days after opening the original bottle, and from another sample of Lyovac, five, twelve, and one hundred fifty days after opening. Ten milliliters of each plasma sample were injected into rabbits. The results (Table III) suggest that the same amount of aged plasma produced less anemia and thrombocytopenia than it did when first reconstituted, and that antired-cell effect appeared to be lost more rapidly than the thrombocytopenic effect. No differences between heated and unheated plasma of the same age were apparent.

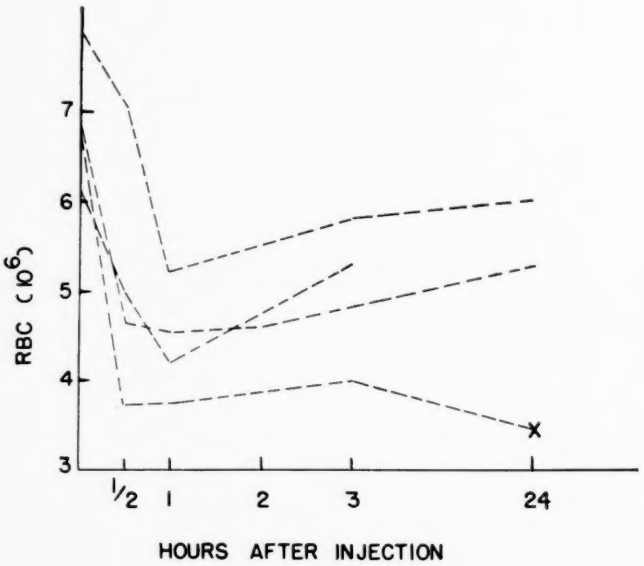

Fig. 2.—Effect of Lyovac on rabbit red-cell levels.

Experiment IV. In Vitro Hemolysis and Agglutination of Rabbit Erythrocytes.—Normal rabbit erythrocytes were suspended in 2 per cent concentration in serial saline dilutions of lyophilized human and fresh dog and sheep plasma. Agglutination occurred up to high titers, as well as hemolysis (Table IV). With the same plasmas heated for fifteen minutes at 56° C. and centrifuged free of precipitate, there was some diminution of agglutination, and hemolysis was not observed since complement was not replaced. With plasma aged for one hundred fifty days there was an appreciable decrease in activity. The erythrocytes of one rabbit which had received repeated inoculations of Lyovac exhibited no increase in the titer of agglutination or hemolysis. Dog and sheep plasmas agglutinated rabbit cells only in the lower dilutions, the dog plasma showing greater hemolyzing tendency.

Experiment V. Agglutination of Platelets in Vitro.—One volume of platelet-rich plasma, prepared by centrifuging normal rabbit blood collected in $\frac{1}{10}$ volume of sodium citrate at 2,000 r.p.m. for 90 seconds, was diluted in white cell pipettes with 19 volumes of fresh human plasma, Lyovac, and samples of plasma from two patients with idiopathic thrombocytopenic purpura (ITP) obtained

TABLE II. EFFECT OF SERUM OR PLASMA ON RABBIT RED-CELL LEVELS

RABBIT NO.	DOSE	R.B.C. (10^6) INITIAL	½ HR.	1 HR.	3 HR.	24 HR.
12	10 ml. Lyovac	6.83	4.67	4.58	4.62	5.32
19	20 ml. Lyovac	6.76	3.78	3.79	4.01	x
55	10 ml. Lyovac	6.51	5.95	5.36	5.90	
57	5 ml. ITP serum	6.79	6.12	6.14	6.38	
2′	10 ml. dog plasma	6.50	6.40	5.60	5.24	
26	10 ml. dog plasma	7.55	7.51	6.27	6.37	7.24
4′	10 ml. sheep plasma	6.56	6.43	6.48	5.86	
20	10 ml. sheep plasma	7.09	6.80	5.22	5.76	7.41

TABLE III. EFFECT OF HEATED AND AGED LYOPHILIZED PLASMA ON RABBIT BLOOD COUNT

RABBIT NO.	ALL DOSES 10 ML. MATERIAL	PLATELETS (10^3)/R.B.C. (10^6) INITIAL	½ HR.	1 HR.	3 HR.	24 HR.
12	Fresh Lyovac$_1$	674 (788) 6.83	106 (60) 4.67	101 (79) 4.58	293 4.62	310 (370) 5.32
7	Lyovac$_2$, 4 days old	469 6.9	266 (240) 7.12	274 (275) 6.37	295 6.26	287 5.94
17	Lyovac$_2$, 14 days old	458 6.42	352 (284) 6.77	343 (308) 7.00	282 (355) 6.27	
157	Lyovac$_3$, 5 days old	846 (780) 6.06	707 (675) 5.01	455 (569) 4.26	623 (693) 5.32	
25	Lyovac$_3$, 12 days old	521 (604) 7.86	333 (280) 7.07	348 (270) 5.26	273 (300) 5.82	347 (426) 6.02
200	Lyovac$_3$, 150 days old	846 6.27	704 6.38	641 6.31	588 5.99	
6	Lyovac$_2$, 4 days old (heated)	497 6.56	409 (345) 6.43	265 (350) 6.48	491 5.86	
21	Lyovac$_2$, 14 days old (heated)	484 6.66	366 (364) 6.48	422 (355) 6.69	410 (376) 6.45	
158	Lyovac$_3$, 12 days old (heated)	746 (700) 6.55	488 (557) 6.98	638 (647) 6.54	595 (635) 6.75	

Arterial platelet counts in parentheses.

TABLE IV. AGGLUTINATION OF RABBIT RED CELLS BY PLASMA IN VITRO

MATERIAL		TITER OF PLASMA 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Lyophilized plasma	Agg.	++++	++++	++++	++++	++++	+++	+	±	0
	Hemol.	++	+	+						
Heated lyophilized plasma	Agg.	++++	++++	++++	++++	+++	++	±	0	
150-day-old lyophilized plasma	Agg.	++++	++++	+++	++	+	±	0		
	Hemol.	+	±							
Dog	Agg.			+	+	+	0			
	Hemol.	++++	++++	+++	+	0				
Dog (heated)	Agg.	+++	++	+	0					
Sheep	Agg.	++	++	+++	+	±				
	Hemol.	+++	++	+	0					
Sheep (heated)	Agg.	++++	+++	+	±	±				
Unheated plasma (red cells of previously injected rabbit)	Agg.	++++	++++	++++	++++	+++	++	+	±	0
	Hemol.	++	++	+						
Red cells of rabbit which had previously received lyophilized plasma, 2 c.c. daily for 8 days	Agg.		+++	+++	+++	+++	++	+		
	Hemol.	++++	+++	+						

TABLE V. FAILURE OF ACTH, CORTISONE, BENADRYL, NEO-ANTERGAN, AND SPLENECTOMY TO PROTECT RABBITS AGAINST THE ANTICELLULAR ACTION OF NORMAL PLASMA

RABBIT NO.	TREATMENT	PLATELETS (10^6)/R.B.C. (10^6)						
		INITIAL	½ HR.	1 HR.	2 HR.	3 HR.	24 HR.	48 HR.
6	ACTH	600	420		200	150	Died at 6 hr.	
	+ 3 ml. plasma							
22	Cortisone acetate	498	512 (500)	428 (425)		(450)	740 (655)	
	+ 10 ml. plasma	5.99	4.90	5.27		4.93	5.23	
11	Cortisone acetate	462 (573)	278 (328)	270 (380)		394 (498)		448 (467)
	+10 ml. plasma	6.01	4.81	5.78		5.96		5.23
27	Cortisone acetate	990	624 (720)	488 (540)		520 (560)		540 (525)
	+10 ml. plasma	5.84	5.74	5.01		5.20		5.41
31	Cortisone acetate	552 (540)	438 (475)	302 (380)		437 (525)		
	+ 5 ml. plasma	5.95	6.37	5.61		6.42	5.49	
41	Benadryl	689 (627)	379 (362)	368 (462)		495 (473)		
	+ 10 ml. plasma	7.40	6.47	6.94		5.78		
44	Neo-Antergan	613 (647)	348 (386)	388		465 (482)		
	+ 10 ml. plasma	6.08	5.58	6.08		5.63		
25	Splenectomized	607 (575)	224 (302)	368		340 (354)	318 (374)	
	10 ml. plasma	5.05	5.82	4.61		4.73	4.70	
29	Splenectomized	715 (684)	252 (326)	430 (428)		457 (482)		
	10 ml. plasma	7.00	7.09	6.50		5.84		

Arterial platelet counts in parentheses.

several days previously. The mixtures were allowed to stand one hour at room temperature, then placed in hemocytometers and examined microscopically for agglutination. There was an occasional small clump in all mixtures except in one of the two ITP plasmas which showed marked agglutination, swelling, and lysis of the platelets. After being suspended in this particular ITP for thirty minutes, the suspension of agglutinated rabbit platelets was passed through a filter of glass wool prepared according to the method of Moolten and Vroman.[5] Platelets thus treated showed no tendency to be filtered out in the Moolten filter, in spite of being visibly agglutinated under the microscope.

Experiment VI. Failure of ACTH, Cortisone, Antihistamines, and Splenectomy to Prevent the Anticellular Action of Plasma.—A rabbit injected with 5 mg. of ACTH three times daily for three days prior to injection with 3 ml. of normal human plasma showed no apparent protective action of the ACTH against the usual fall of platelets and red cells (Table V). Three rabbits injected with doses of 10 mg. of cortisone acetate per kilogram of body weight twenty-four hours and one hour prior to injection, and 2 rabbits injected, respectively, with Neo-Antergan and Benadryl in doses of 5 mg. per kilogram body weight 1 hour before, and 10 mg. per kilogram of body weight immediately preceding, injection with 10 ml. of lyophilized plasma likewise received no protection. In two rabbits previously splenectomized, 10 ml. of lyophilized plasma had just as great an effect as in the intact animals.

Experiment VII. Effect of Repeated Injection of Plasma.—Repeated isolated injections at intervals of seven and forty days did not appear to protect against, or enhance the effect and did not produce a progressive thrombocytopenia in 2 rabbits. Repeated daily injections for forty days did appear to confer tolerance, as evidenced by the increasing ineffectiveness of graduated daily doses from 1 to 10 ml. of lyophilized plasma in one rabbit. Some of this effect may have been due to attenuation of the plasma by aging.

Experiment VIII. Separation of the Antiplatelet Factor From Normal Dog Plasma, and ITP Serum.—Fresh dog plasma was precipitated with $Ca_3(PO_4)_2$ gel[6] and the precipitate was eluted with 0.2 M sodium citrate solution. In two rabbits 10 ml. of the plasma thus treated was found to retain its antired-cell property on injection but to have lost most of its antiplatelet effect (Table VI). The eluate, injected into two rabbits in 10 ml. quantities, produced a marked thrombocytopenia, but had no effect on the red blood cells. One-week-old serum from a patient with idiopathic thrombocytopenia was similarly treated and the eluted serum and eluate were injected into rabbits with respectively similar results.

DISCUSSION

These studies indicated that thrombocytopenia and anemia could be produced in the blood of rabbits by the injection of heterologous serum or plasma. In most of the animals an initial lymphopenia and subsequent leukocytosis were also observed, but these were inconsistent and probably represent an "alarm" reaction to the injection, mediated through the adrenal cortex. The

TABLE VI. EFFECT OF $Ca_3(PO_4)_2$ GEL-TREATED DOG PLASMA AND ITP SERUM ON RABBIT CELLS

RABBIT NO.	DOSE AND MATERIAL	PLATELETS (10^3)/R.B.C. (10^6)				
		INITIAL	½ HR.	1 HR.	3 HR.	24 HR.
41	10 ml. treated dog plasma	540 6.20	492 (512) 6.45	501 (470) 5.47	487 5.20	526 (509) 5.97
72	10 ml. treated dog plasma$_2$	424 (570) 6.04	592 (550) 5.82	507 (402) 5.53	584 (609) 4.76	
44	10 ml. eluate$_1$	491 5.78	219 (248) 5.95	296 (224) 6.16	458 (397) 5.83	392 (460) 5.36
42	10 ml. eluate$_2$	527 (530) 7.14		385 (364) 6.50	 6.82	379 (460) 6.67
57	5 ml. ITP serum	626 (680) 6.79	412 (450) 6.12	415 (380) 6.14	595 6.38	
58	5 ml. treated ITP serum	512 (466) 6.27	450 6.21	472 (448) 5.62	465 6.12	
61	5 ml. sodium citrate eluate of treated ITP serum	692 (680) 5.94	392 (480) 5.88	519 (491) 5.69	467 5.72	

Arterial blood platelet counts in parentheses.

antiplatelet and antired-cell effects were apparent one-half to one hour following the injection and lasted from twenty-four to forty-eight hours. They were not prevented by splenectomy or treatment with ACTH, cortisone, or antihistamine drugs.

Stefanini and associates[3] noticed in rabbits the same phenomenon, which we have described, as well as a transient thrombocytopenic effect in man following the transfusion of blood or plasma,[6] which was due to a factor in plasma, likewise separable by treatment with $Ca_3(PO_4)_2$ gel. Bierman and associates[7] observed that the arterial platelet levels in such instances lagged several hours behind the venous levels, which they interpreted as evidence that the factor acted on the platelets in the peripheral capillary bed. In our observation on rabbits no differences between arterial and venous platelet levels were observed. Whether or not the factor responsible for the depression of platelet levels in man is identical with the factor we have studied in the rabbit is debatable.

The precipitation procedure with $Ca_3(PO_4)_2$ and subsequent elution with citrate solution, which separates the antiplatelet factor, is known to remove prothrombin, some A-C globulin, and serum prothrombin conversion accelerator (SPCA).[8] Prothrombin (or the thrombin formed from it) may be responsible for platelet agglutination in vitro in serum but not in plasma[9] and might be responsible for the in vivo effects we observed with serum. It does not appear likely that such similar effects obtained with plasma and serum would be due to different factors, and, since the plasma effects are probably not due to prothrombin, it appears more probable that the thrombocytopenic factor is something else that is removed with the prothrombin. Studies are in process to investigate this point further.

The existence of interspecies effects in erythrocytes has been known since the days of Landois,[10] but the coexistence of similar effects on platelets has received less attention. Their presence in different species gives no support for an acquired immunologic mechanism for their production.

The antired-cell factor is classically regarded as a species-specific hemagglutinin or "natural antibody." This does not, however, necessarily mean that the antiplatelet factor is likewise, although recent evidence[11] adds strength to the idea that it might be. That acquired antiplatelet substances can be formed which agglutinate platelets in the right environmental conditions is shown by the studies of Ackroyd[12] and of Bigelow and Desforges[11] with reference to the thrombocytopenia produced by sensitivity to Sedormid and quinidine, respectively. Stefanini and associates[14] have reported detailed observations of a case of idiopathic thrombocytopenic purpura with a platelet agglutinin possessing characteristics of an antibody. The observations of Hirsch and Gardner[15] that successive platelet transfusions in patients with aplastic anemia showed progressively shorter survivals of the transfused platelets suggest the formation of an antiplatelet substance. There is increasing awareness of the antigenic similarities between many nonspecific antigens and those of erythrocytes,[16] and the interaction of bacterial antigens such as *Vibrio cholerae* with naturally occurring agglutinins in normal blood to form new antigens capable of inducing the formation of antibodies specific for red cells "treated" in vitro with the bacteria in question,[17] or the immunologic modification of red cells by viruses,[18] suggests possible mechanisms of production of auto-agglutinins and hemolysins in acquired hemolytic anemias. Motulsky[19] has recently reported that influenza virus can also be adsorbed onto the surface of platelets, which suggests that similar mechanisms may operate in the pathogenesis of idiopathic thrombocytopenic purpura.

In view of the current interest in immune pathogenic mechanisms in idiopathic thrombocytopenia, the existence of such naturally occurring antiplatelet substances as we have demonstrated is noteworthy, if only in providing a tool for the ready production of thrombocytopenia in rabbits. The possibility that the activity of such substances may be enhanced by nonspecific immune reactions coexisting independently, bringing about a destruction of platelets that initiates the formation of more specific autoplatelet agglutinins, is submitted, in the absence of specific proof, as a working hypothesis to explain the pathogenesis of idiopathic thrombocytopenic purpura.

SUMMARY

Normal human, dog, and sheep plasma or serum contains antirabbit platelet and erythrocyte factors which produce thrombocytopenia and anemia in rabbits following intravenous injection. These factors are relatively stable to heat, but labile on aging. Their effect is not opposed by ACTH, cortisone, antihistaminics, or splenectomy. The antiplatelet factor can be separated from the plasma by chemical means that also remove prothrombin.

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STUDIES ON PLATELETS

XI. Antigenicity of Platelets and Evidence of Platelet Groups and Types in Man

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THE administration of heterologous platelets to animals results in the production of specific antiplatelet serum. "Immune" serum causes marked thrombocytopenia when injected into the circulation of the donor animals. Such experiments, performed by Marino[1] as early as 1905, established the heteroantigenicity of blood platelets.

More recent observations suggest the iso- and autoantigenicity of blood platelets as well. A thrombocytopenic factor has been demonstrated in the plasma of some patients with idiopathic thrombocytopenic purpura,[2] and platelet iso- and autoagglutinins have been detected in the plasma of these patients.[3-7] Further and, perhaps, more direct evidence for platelet iso- and autoantigenicity has been obtained in the course of platelet transfusion studies. Patients with secondary or "amegakaryocytic" thrombocytopenic purpura show progressively less benefit from repeated platelet transfusions.[8-11] At the same time, they may develop iso- and, occasionally, autoplatelet agglutinins,[10, 11] and their plasma may induce thrombocytopenia when injected into normal recipients.[11]

The demonstration that platelets are antigenic suggests the existence of individual platelet groups and types. In 1923, Toda[12] tried to investigate the presence of different antigenic structures in platelets by the use of anti-red cell human sera. He failed, however, to observe agglutination of platelets in a limited number of experiments. More recently, Harrington and associates[5, 7] have discussed the existence of different platelet types. Eight individual types were established by these authors,[7] and one was clearly identified by absorption studies in vitro and immunization studies in vivo. Evidence for the existence of several platelet groups and types in man is presented in this article.

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This work was supported by grants from the National Institutes of Health, USPHS, the American Heart Association, and was completed under contract (1276) with the Atomic Energy Commission.

Presented in part at the Forty-fifth Annual Meeting of the American Society for Clinical Investigation, May 4, 1953, Atlantic City, N. J.

Received for publication, May 5, 1953.

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MATERIALS AND METHODS

(A) *Detection of Platelet Agglutinins.*—Among many techniques, the following was found most satisfactory:

Reagents: (1) Platelet-rich plasma was prepared as follows: blood was collected in silicone-coated glassware (double syringe technique), through Arquad 2-C coated needles from healthy donors. One-tenth volume of Sequestrene-Na_2 1 per cent in 0.7 per cent NaCl was the anticoagulant solution. The blood was centrifuged at 1,000 r.p.m. for fifteen minutes in a refrigerated centrifuge at 4° C. The supernatant platelet-rich plasma was transferred with silicone-coated pipettes to another silicone-coated test tube. The remaining blood was then centrifuged for thirty minutes at 3,000 r.p.m. at 4° C., and the platelet-poor plasma obtained was used to adjust the platelet count of the platelet-rich plasma to 200,000 per cubic millimeter as determined by a direct method.[13] The final preparation of platelets was stored at 4° C. and used within five hours of collection. Alternatively (for detection of complete antibodies) platelet suspensions in saline prepared as described[10] and adjusted to a final count of 200,000 per cubic millimeter were used.

(2) Platelet-poor plasma was used as the source of the agglutinin. Blood was collected from the individuals studied as described in (1) and immediately centrifuged at 3,000 r.p.m. for thirty minutes at 4° C. The supernatant, platelet-poor plasma (containing at the most 5,000 to 10,000 platelets per cubic millimeter) was used within five hours of collection. In some instances it was quickly frozen, stored at −20° C. and used within five hours after thawing. It was found occasionally, however, that thawed plasma would be able to agglutinate a larger number of platelet samples than when freshly collected. For this reason, most of the experiments were conducted with plasma tested within five hours of collection.

Technique: Equal volumes (0.1 ml.) of platelet suspension in plasma (or in saline solution) and platelet-free plasma being studied for agglutinins were transferred to a silicone-coated test tube, the mixture incubated for sixty minutes at room temperature and then centrifuged at 500 r.p.m. for two minutes. After resuspending with gentle tapping, the mixture was poured over a silicone-coated slide,* and covered without applying pressure with a chemically clean cover slip. Agglutination was read under ×430 magnification (high dry objective lens of the microscope and 10× eyepiece). It was graded from 1 to 3+ depending on the number of platelet clumps in each field and their size (Fig. 1). Several techniques were tried to improve the sensitivity of the test. Acidification of plasma with citric acid,[7] trypsinization of platelets, addition of splenic extracts, all failed to make the test more sensitive. Normal, nonagglutinating Sequestrene-Na_2-treated, platelet-poor plasma was used to dilute the agglutinating plasma when the titer of the latter was to be determined.

(B) *Agglutinin Absorption Technique.*—Platelet buttons were prepared by centrifuging at high speed (3,000 r.p.m. for thirty minutes at 4° C.) platelet-rich plasma from 20 ml. of blood. The button was washed with saline solution twice. To absorb the agglutinin, 1 ml. of plasma was transferred to a test tube containing a platelet button; the platelets were resuspended by stirring with a silicone-coated glass stirring rod and the mixture incubated at 37° C. for thirty minutes. The supernatant was then separated by centrifugation at 3,000 r.p.m. for fifteen minutes. The procedure was repeated until the supernatant was no longer able to agglutinate the original platelet suspension. Two to four absorptions at least were necessary in each case.

(C) *Stimulation of Agglutinating Titer In Vivo.*—Repeated transfusions of "incompatible" platelets were administered to individuals whose plasma demonstrated some or no agglutinating activity against certain platelet samples. Two techniques were used:

*Slides treated with 10 per cent silicone in petroleum ether, repeatedly washed with tap and distilled water, then left under distilled water until the moment of use, when they were carefully dried with lint-free gauze.

small intravenous injections of 25 to 50 ml. of platelet-rich plasma, weekly for six to eight weeks; large transfusions (500 ml.) at intervals of four to six weeks. The agglutinating titer of the platelet recipient was followed at regular intervals with the procedure described above.

(D) *Preparation of Anti-Human Platelet Plasma in Rabbits.*—Albino rabbits, of 6 pounds average weight, were given at weekly intervals plasma-free suspensions of platelets in saline solution prepared from 40 ml. of blood from the same human donor. The rabbit plasma was originally unable to agglutinate human platelets. An antihuman platelet agglutinin developed, however, within a three-week period, with a titer of 1/32 to 1/128 in single cases. Each plasma was then absorbed twice with saline-washed group O platelets of human origin, as described in (B). The absorbed plasmas were then tested against various samples of human platelets in plasma.

(E) Administrations of "immune" plasma, "incompatible" plasma, human platelet-rich plasma and of various preparations of platelets for determination of platelet survival time were performed as indicated in previous communications.[14-16]

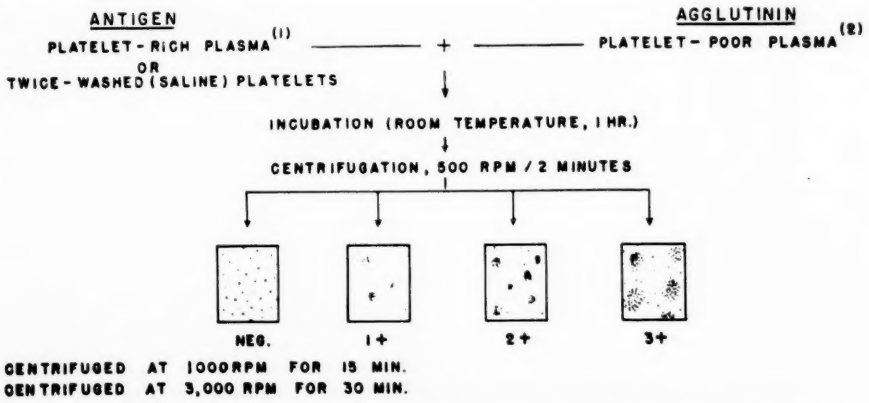
ANTIGEN
PLATELET-RICH PLASMA (1)
OR
TWICE-WASHED (SALINE) PLATELETS
AGGLUTININ
PLATELET-POOR PLASMA (2)
INCUBATION (ROOM TEMPERATURE, 1 HR.)
CENTRIFUGATION, 500 RPM / 2 MINUTES
NEG.
1+
2+
3+
(1) CENTRIFUGED AT 1000RPM FOR 15 MIN.
(2) CENTRIFUGED AT 3,000 RPM FOR 30 MIN.
ALL GLASSWARE COATED WITH SILICONE; BLOOD COLLECTED THROUGH ARQUAD 2-C NEEDLES IN SILICONE-COATED SYRINGE; 1/10 SEQUESTRENE-Na2 (0.7 TO 1%) USED AS ANTICOAGULANT. ACIDIFICATION OF PLASMA, TRYPSINIZATION OF PLATELETS OR ADDITION OF SPLENIC EXTRACT DO NOT ENHANCE AGGLUTINATION.

Fig. 1.—Detection of agglutinins in "grouping and typing" of platelets.*

RESULTS

I. *General Evidence for the Existence of Platelet Groups and Types.*—

(A) *Development of platelet agglutinins in patients with "amegakaryocytic" thrombocytopenic purpura receiving multiple platelet transfusions:* During the past three years, we have administered three or more transfusions of variously prepared platelets to thirty-six patients with "amegakaryocytic" or secondary thrombocytopenic purpura.[15] Of these patients, 21 (58 per cent) showed progressively decreasing response to the hemostatic effect of platelet transfusions and shorter survival of transfused platelets. Seventeen patients (47 per cent) developed platelet agglutinins in titers varying from 1/2 to 1/32. The plasma of six patients (capable of agglutinating platelets in vitro at a titer of 1/16 or higher) induced thrombocytopenia when transfused into nor-

*One per cent Sequestrene-Na_2 in 0.7 per cent NaCl was used in most experiments. The concentration of the anticoagulant was lowered to a minimum figure of 0.7 per cent Sequestrene-Na_2 in 0.7 per cent NaCl in few experiments to reduce the anticomplementary activity of Sequestrene.

mal recipients (Table I). This finding is in agreement with previous observations that platelet agglutinin titer in vitro and thrombocytopenic effect in vivo are closely correlated in patients with idiopathic thrombocytopenic purpura who exhibit platelet agglutinins.[14, 7] Many of the patients studied were receiving ACTH and/or cortisone while transfused. Thus, the use of the hormones seemed unable to prevent the development of platelet agglutinins (Table I).

TABLE I. DEVELOPMENT OF PLATELET AGGLUTININ, THROMBOCYTOPENIC EFFECT OF PLASMA AND RESISTANCE TO PLATELET TRANSFUSION IN 22 OF 36 PATIENTS OF "AMEGAKARYOCYTIC" PURPURA RECEIVING MULTIPLE PLATELET TRANSFUSIONS

	PATIENT,	SEX,	AGE	CLINICAL DIAGNOSIS	PLATELET AGGLUTININ, TITER	RESISTANCE TO PLATELET TRANSFUSIONS	THROMBOCYTOPENIC EFFECT OF PLASMA	THERAPY
1	J. F.,	M,	16	Lymphocytic leukemia (acute)	1/4	+	None	ACTH
2	S. S.,	M,	73	Hypoplastic anemia		+	None	Cortisone
3	I. N.,	M,	51	Aplastic anemia	1/32	+	+	ACTH
4	S. P.,	F,	19	Hypoplastic anemia	1/16	+	+	ACTH
5	J. C.,	M,	60	Metastatic carcinoma (prostate)	1/16	+	+	Cortisone
6	S. W.,	F,	55	Lymphocytic leukemia	1/4	+	None	
7	I. B.,	M,	49	Aplastic anemia	1/8	+	None	
8	H. S.,	M,	47	Metastatic carcinoma (lung)	1/4	+	*	
9	J. D.,	M,	35	Hypoplastic anemia	1/4	+	None	
10	D. J.,	F,	12	Aplastic anemia		+	None	
11	W. M.,	F,	14	Aplastic anemia	1/8	+	*	ACTH
12	B. M.,	F,	34	Hypoplastic anemia	1/16	+	+	
13	D. S.,	M,	45	Hypoplastic anemia	1/2	+	None	Cortisone
14	M. R.,	M,	3	Lymphocytic leukemia (subacute)	1/16	+	+	ACTH
15	A. R.,	F,	13	Lymphocytic leukemia (subacute)	1/4	+	None	ACTH
16	W. E.,	F,	63	Lymphocytic leukemia (subacute)	1/8	+	*	ACTH
17	M. D.,	F,	4	Lymphocytic leukemia (subacute)		+	None	ACTH
18	S. DiP.,	M,	43	Hypoplastic anemia	1/4	+	None	Cortisone
19	M. H.,	F,	42	Hypoplastic anemia	1/16	+	+	ACTH
20	M. D.,	F,	35	Granulocytic leukemia		+	None	ACTH
21	S. D.,	M,	59	Lymphosarcoma	1/4	+	None	Nitrogen mustard

*Not determined.

(B) *Development of platelet agglutinins in nonthrombocytopenic patients receiving multiple transfusions:* During the past three and one-half years, 26 patients who did not present thrombocytopenia received multiple transfusions of platelet-rich blood or plasma for various reasons. The plasma of 9 of these patients was found to agglutinate platelets. One case, a hemophiliac (Fig. 2), *developed thrombocytopenia* and resistance to the hemostatic activity of antihemophilic globulin. His plasma agglutinated platelets from many donors and the patient's own platelets.

Platelet isoagglutinins were also detected in nine instances in a group of 295 patients who had received multiple transfusions of blood and plasma col-

lected and administered without consideration of platelet preservation. The nine included 2 patients with Mediterranean anemia, 2 with cirrhosis of the liver with repeated hematemesis, 1 with hemophilia, 1 with "fibrinolytic purpura" due to disseminated carcinoma of the prostate, 1 with paroxysmal nocturnal hemoglobinuria, and 2 with aplastic anemia. The development of platelet agglutinin in a patient of paroxysmal nocturnal hemoglobinuria who had received only transfusions of saline-washed red cells to prevent transfusion reactions cannot be satisfactorily explained.

Comparing the figures of the last paragraph and those of the first paragraph and section (A), we have been impressed by the higher incidence of platelet agglutinins in the group of patients transfused with "viable" platelets (blood or plasma collected in silicone-coated bottles or in plastic bags). This finding raises the question as to whether well-preserved platelets are more antigenic than damaged platelets and platelet products collected in glass bottles. That this may be true is also suggested by the observation that as few as 2 to 3 transfusions of well-preserved platelets were often followed by the development of platelet agglutinins in thrombocytopenic recipients.

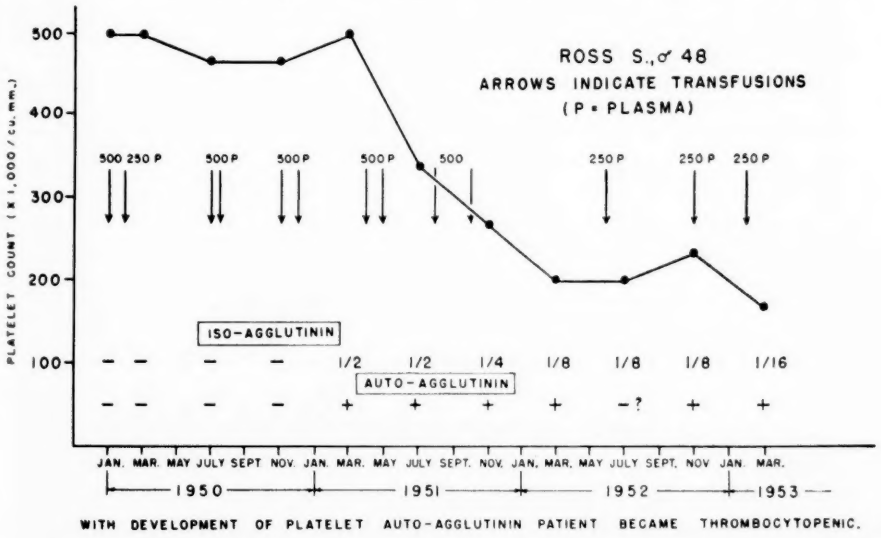

Fig. 2.—Development of iso- and auto-platelet agglutinin and thrombocytopenia in a hemophiliac receiving multiple blood and plasma transfusions. (This patient also developed increased resistance to the hemostatic effect of transfusions.)

(C) *Platelet agglutinins in the plasma of patients with idiopathic thrombocytopenic purpura:* Detection of platelet agglutinins in the plasma of patients with idiopathic thrombocytopenic purpura has been reported by several authors as well as by us.[3-7] In our series of 78 cases of idiopathic thrombocytopenic purpura, platelet agglutinins were detected in a total of one case of the "acute" variety and 21 of the "chronic" variety[17] (Table II). The presence of platelet agglutinins was accompanied in 15 cases by the ability of the patient's plasma to induce thrombocytopenia in a normal recipient. This

was extremely pronounced and prolonged (seven to eight days) in only two cases, with platelet agglutinin titers of 1/2048 and 1/512, respectively. Table II shows that, in 13 instances, an autoagglutinin was also present. In 2 instances, an autoagglutinin represented the only antiplatelet factor detected in the patient's plasma.* The antiglobulin technique previously described[14] detected a "coating" agglutinin in 6 cases.

TABLE II. PRESENCE OF PLATELET ISOAGGLUTININS AND AUTOAGGLUTININS, THROMBOCYTOPENIC EFFECT OF PLASMA AND SURVIVAL OF INJECTED PLATELETS IN 22 OF 78 CASES OF IDIOPATHIC THROMBOCYTOPENIC PURPURA

	PATIENT, SEX, AGE	AUTO-AGGLUTININS	ISO-AGGLUTININS	COATING AGGLUTININS	THROMBOCYTOPENIC EFFECT OF PLASMA	PLATELET SURVIVAL	RESULT OF SPLENECTOMY
1	A. G., F, 58	1/4	1/4	+	±	4 hours	Good
2	E. L., F, 57	1/2	1/8	0			
3	F. S., F, 25	1/4	1/16	+	+		Negative
4	P. S., F, 5	"acute"	1/2				
5	M. S., F, 52		1/8	0		3 hours	
6	B. S., M, 11	1/16	1/8	0	±	1 hour	Good
7	A. R., F, 44	1/2	1/8	+			
8	M. L., F, 42		1/2	0	±	12 hours	Good
9	M. R., F, 28		1/2	0	0	18 hours	Good
10	C. I., F, 39	1/2	1/4	0	0	8 hours	
11	H. E., F, 48	1/16	1/16	+	+	10 hours	Good
12	J. B., M, 52		1/4		±	2 hours	Negative
13	L. Z., M, 57	1/512	1/256	+	++	2 hours	Negative
14	R. P., M, 10		1/8	0	±	4 hours	Good
15	T. T., F, 83		1/16	0	±	12 hours	
16	M. K., F, 59	1/2048	1/2048	++	+++	0 hours	Negative
17	J. C., F, 14		1/2		±	4 hours	Good
18	G. T., F, 17	1/8	1/8	0	±	12 hours	Good
19	M. H., F, 56		1/16		+		Negative
20	D. McG., F, 21	1/8	1/16	0	+	6 hours	Good
21	K. B., F, 38	1/8	0	0	+	12 hours	Negative
22	M. P., F, 27	1/2	0	0	0		

Direct serologic techniques and absorption studies indicated that, with two exceptions only, the agglutinin would be active against all samples of platelets ("panagglutinin"). Negative agglutination tests would at times become positive if careful consideration was given to the optimum proportion between number of platelets in the final mixture and titer of the agglutinin (Table III). In the two cases representing the exception, only some samples of platelets were agglutinated by the patient's plasma. In 12 of 15 cases studied, splenectomy failed to cause disappearance or even diminution in titer of the platelet isoagglutinin. In 5 of 9 cases, however, while the isoagglutinin persisted at practically unmodified titer, the autoagglutinin was found to have disappeared between fifteen and thirty days following splenectomy (Table IV). In all cases, the platelet count had returned to normal or high level in five to ten days.

*Presence of autoagglutinin was tested by mixing the plasma of the patient collected at the time of maximum thrombocytopenia and kept under frozen storage with the platelets collected at the time of partial or complete remission. Also, by examining plasma separated from the patient's blood by slow-speed centrifugation at the time of severe thrombocytopenia, it was possible, at times, to see few platelets collected in small clumps. This phenomenon, however, was too inconstant to be utilized as evidence for platelet autoagglutinins.

TABLE III. IMPORTANCE OF OPTIMUM PROPORTION BETWEEN NUMBER OF PLATELETS IN THE FINAL PREPARATION AND CONCENTRATION OF THE AGGLUTININ FOR THE POSITIVITY OF THE AGGLUTINATION TEST AGAINST DIFFERENT PLATELET SAMPLES

NUMBER OF PLATELETS IN FINAL MIXTURE* PER C.MM.	25,000	50,000	75,000	100,000	200,000	300,000
Preparation of platelet, donor						
1	−	+	+	+	+	+
2	−	+	+	+	−	−
3	−	−	+	+	+	+
4	−	−	−	+	+	+
5	+	+	+	+	+	+
6	−	−	+	+	+	+
7	+	+	+	+	+	+
8	−	−	−	+	+	+
9	+	+	+	+	−	−
10	−	−	+	+	+	+

Patient: A, G., 58-year-old woman, "chronic idiopathic thrombocytopenic purpura." Platelet isoagglutinin, titer against most platelets: ¼.
*0.1 ml. of platelet suspension in plasma + 0.1 ml. of platelet-poor patient's plasma.

TABLE IV. EFFECT OF SUCCESSFUL SPLENECTOMY ON THE TITER OF ISO- AND AUTOPLATELET AGGLUTININS IN FIVE PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA ("CHRONIC" VARIETY)

	AUTOAGGLUTININ		ISOAGGLUTININ	
PATIENT, SEX, AGE	BEFORE SPLENECTOMY	AFTER SPLENECTOMY	BEFORE SPLENECTOMY	AFTER SPLENECTOMY
H. C., F, 46	1/16	neg.	1/32	1/16
D. McG., F, 21	1/8	neg.	1/16	1/8
B. S., M, 11	1/16	neg.	1/8	neg.
G. T., F, 17	1/8	neg.	1/8	1/8
A. G., F, 58	1/4	neg.	1/4	1/4

(D) *Thrombocytopenic effect of normal plasma:* As previously reported,[18] administration of compatible plasma caused transitory and moderate drop in the platelet count of 7 to 8 of 10 normal recipients. This finding was previously considered evidence of platelet antigenicity and of the existence of platelet groups and types. Further work has indicated, however, that the thrombocytopenic effect of normal plasma is probably unrelated to platelet agglutinins or platelet incompatibility.[19]

II. *Specific Evidence for the Existence of Platelet Groups and Types in Man.*—

This was sought for by the following techniques, which have been described in the section on Materials and Methods:

– agglutination of platelets by normal plasmas (naturally occurring agglutinins);

– agglutination of platelets by "immune" plasmas (acquired agglutinins);

– agglutination of platelets by "absorbed" immune heterologous plasmas.

(A) *Agglutination of platelets by plasmas of normal, previously untransfused individuals (platelet groups):* Platelets and plasmas of 215 healthy individuals, never previously transfused, never pregnant, and not receiving medication of any kind were cross-tested. Of these plasmas, 13 (approximately 6 per cent) showed ability to agglutinate consistently the platelets from some

of the donors. Platelets from 41 individuals (approximately 19 per cent) were consistently agglutinated by one or more of the plasmas (Table V). Analysis of the data (including over 55,000 agglutination tests) revealed the probable presence of two agglutinins which may be designated as anti-I and anti-II, the corresponding antigens being designated as I and II. The incidence of each antigen in this series of normal individuals is given in Table V. Four platelet groups are then probably present: I, II, III (I + II) and IV (O). Many of the agglutination tests were run in duplicate, and, as suggested by Dr. Alexander S. Wiener, using the "blind test" technique; consistent results were obtained. The designation of the two platelet group antigens as A, B, AB, and O has also much to recommend itself, provided it is clearly understood that this classification in no way relates platelet to red-cell groups.

TABLE V. DESIGNATION OF PLATELET GROUPS AND THEIR INCIDENCE AMONG THE GENERAL POPULATION (285 INDIVIDUALS)

PLATELET GROUPS	I	II	III (I + II)	IV (O)
Platelet agglutinin				
-anti-I (3.5 per cent)	+	–	+	–
-anti-II (2.5 per cent)	–	+	+	–
Incidence, per cent	10.2	4.6	3.7	81.5

Further demonstration of platelet groups was made by agglutinin absorption techniques. Absorption of plasma containing agglutinin anti-I with platelets of group I or III, and of plasma containing agglutinin anti-II with platelets of group II or III, respectively, left a plasma incapable of agglutinating any platelet sample. In vivo studies also offered parallel conclusions. A recipient of platelet group O and nonexhibiting agglutinins was given, weekly, 25 ml. of group I platelet-rich plasma. Eight weeks later, his plasma would agglutinate platelets of group I donor at a titer of 1/8. Moreover, at the beginning of the experiment the administration of 250 ml. of the recipient's plasma into the donor would cause only mild and transitory thrombocytopenia. Eight weeks later, the thrombocytopenia induced with a similar experiment was much more marked and prolonged (Fig. 3). A recipient of platelet group IV (O) whose plasma contained a low-titer (1/4) anti-I platelet agglutinin, was given weekly intravenous injections of 25 ml. of platelet-rich plasma from a platelet group I donor. His anti-I platelet group titer had risen to 1/32 after eight weeks. Prior to the study, the administration of 250 ml. of plasma from the recipient to the donor would cause only moderate thrombocytopenia. The thrombocytopenic effect was, however, much greater after the rise in the platelet agglutinin titer (Fig. 4).

Individuals of platelet group IV (O) included approximately 80 per cent of the subjects examined. To break down this large group, the following study was conducted. Sixteen volunteers were divided in 8 groups of one donor and one recipient. The recipient received the platelet-rich plasma from 50 ml. of the donor's blood weekly for eight weeks. At the end of the study, none of the recipients had developed agglutinins against either the donor's

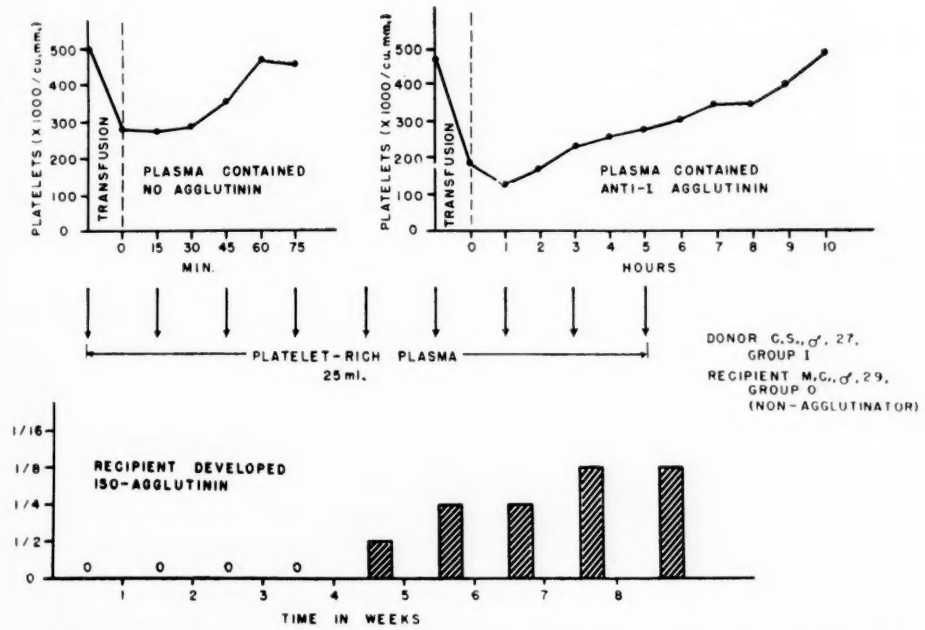

Fig. 3.—Development of platelet agglutinin in an individual receiving group noncompatible platelets. The development of an anti-I agglutinin was accompanied by increased thrombocytopenic effect in vivo of his plasma, when inected into the platelet donor.

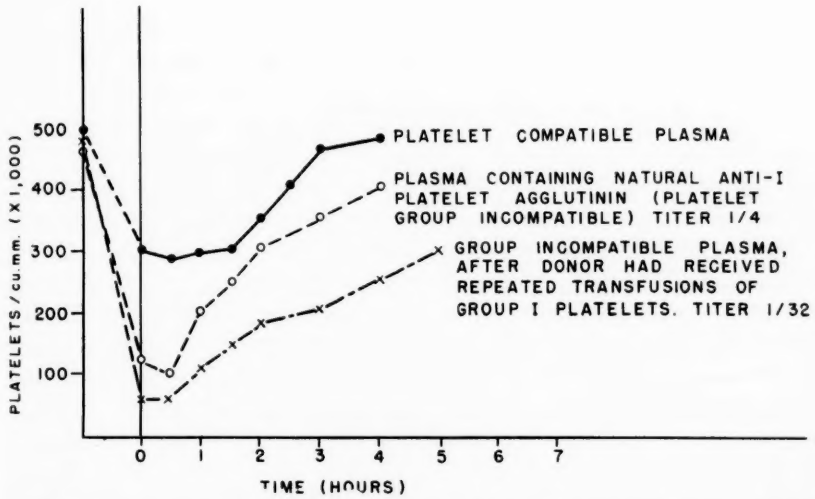
PLATELETS / cu mm. (X 1,000)
PLATELET COMPATIBLE PLASMA
PLASMA CONTAINING NATURAL ANTI-I PLATELET AGGLUTININ (PLATELET GROUP INCOMPATIBLE) TITER 1/4
GROUP INCOMPATIBLE PLASMA, AFTER DONOR HAD RECEIVED REPEATED TRANSFUSIONS OF GROUP I PLATELETS. TITER 1/32
TIME (HOURS)

Fig. 4.—Thrombocytopenic effect in vivo of plasma of individual exhibiting platelet anti-I agglutinin when injected into individual of platelet group I. (Note the increased thrombocytopenic effect of the plasma following repeated transfusions of group I platelets in the individual.)

or any other sample of platelets. This study seems to confirm the homogeneity of this large group of nonagglutinating plasmas and nonagglutinable platelets among the general population.

The 13 agglutinators all belonged to group IV (O). Their agglutinin was probably naturally occurring, since none of the individuals had been pregnant or had been transfused, although it is, of course, possible that the agglutinin could have developed in response to an antigenic stimulus of unknown nature. The agglutinin was equally active against saline or plasma suspensions of platelets. Platelets in saline, however, exhibited as a rule stronger clumping, possibly because the damage caused by their repeated centrifugation and washing had made them more sensitive to the activity of the agglutinin. Plasmas of the individuals whose platelets were agglutinated did not clump platelets of the agglutinating plasma or any other. At least in these experiments, *there was no apparent relationship between red-cell and platelet groups.*

(B) *Agglutination of platelets by plasma of pregnant women:* The plasma from 20 recently delivered healthy women* was tested against the platelets of 28 healthy individuals. In 19 cases, the results were similar to those obtained with normal plasma. Interestingly enough, the plasma of one multipara who had been transfused three times at the termination of two previous pregnancies agglutinated consistently 75 consecutive samples of platelets in saline or plasma. The patient's platelet count was normal and her three children did not show evidence of thrombocytopenia or purpura at birth. The patient's plasma did not agglutinate her own platelets, but clumped those of two of her children tested, at a titer of 1/4. The weakness of the titer may account for the failure to observe thrombocytopenia in the newborn children, if the agglutinin had in effect gained entrance from the maternal to the fetal circulation. Whether the child's platelets could be agglutinated by the child's own plasma collected at birth could not, unfortunately, be studied.

(C) *Agglutination of platelets by "immune" plasma (platelet types):* The plasmas of 15 patients who had developed platelet agglutinins after repeated transfusions were used. Twelve were patients with hypoplastic or aplastic anemia who had received several transfusions of "viable" platelets; one with hemophilia and two with Mediterranean anemia. Six of these plasmas were found to agglutinate 50 consecutive samples of platelets and probably contained platelets "panagglutinins," with a titer from 1/4 to 1/16. Dilution of these plasmas with normal group-compatible plasma in an attempt to detect finer differences in their agglutinating ability did not change the results obtained or, if the dilution was marked, produced negative results.

The remaining 9 plasmas agglutinated some, but not all platelet samples. By using these plasmas as typing plasmas, it was found that only 6 would clump different samples and that, on the basis of agglutination tests, six serologically different types of platelets could be possibly recognized (Table VI). *The definition of "type" was selected because these antigens were revealed by "immune" plasmas, as against "groups" which were revealed by naturally occurring agglutinins.*

*Courtesy of Dr. Arthur J. Gorman, Saint Elizabeth Hospital, Brighton, Massachusetts.

TABLE VI. DESIGNATION OF PLATELET TYPES AND INCIDENCE AMONG THE GENERAL POPULATION (350 INDIVIDUALS)

PLATELET TYPES	1	2	3	4	5	6
Plasma 1*	+	+				+
2	+			+	+	
3	+			+		
4	+				+	
5*	+	+				
6*	+			+		
Incidence, %	34.42	10.28	18.43	18.28	16.16	2.43

*Of nine "immune" plasmas, two behaved like 1, 5, and 6.

The six serologic types were investigated critically with experiments of agglutinin cross-absorption. Each "typing" plasma was absorbed with platelets of each serologic type, and its ability to clump various platelets checked again after absorption. Complete removal of the "immune agglutinin" was difficult and required at least 5 treatments of the plasma with platelet buttons. By this method, only 3 types could be established: 1, 3 and 6. In in vivo studies, platelets of type 1 or 6 were injected into normal recipients of platelet type 3. Platelet-rich plasma from 50 ml. of blood was given weekly for eight weeks. The anti-1 type agglutinin titer, in one experiment, rose to 1/4 after three weeks, to 1/8 after five weeks, and to 1/16 after eight weeks. The recipient's plasma, as in the similar experiment conducted to investigate platelet groups, induced marked and prolonged thrombocytopenia (70 per cent drop for twenty-four hours) when injected into the donor's circulation. A similar result was obtained when a platelet-3 type individual received platelets of type 6. Eight weeks later the recipient's plasma had a 1/4 anti-1 type titer. The immune agglutinin titer was found to decrease slowly after the termination of the study and finally disappear eight to sixteen weeks later. This series of experiments again suggests the existence of at least three distinct platelet types.

(D) *Agglutination of platelets by absorbed heterologous (rabbit) immune plasma:* Several samples of platelets were tested against the plasmas of six rabbits which had been given repeated intravenous injections of washed human platelets. The plasma had been absorbed with human platelets of group IV (O), to remove a specific heterologous agglutinin [section (D) of Materials and Methods]. The purpose of this study was to ascertain the presence of antigens comparable to the M-N system in the red cell. It was found, however, that absorbed immune plasmas were unable to agglutinate any of more than 20 consecutive samples of platelets tested.

DISCUSSION AND CONCLUSIONS

These results are of a preliminary nature and must be interpreted with extreme caution and criticism. While heteroantigenicity and isoantigenicity of platelets are well established, the existence of groups and types in man is suggested by the results obtained by Harrington and associates[7] and by those presented in this paper, but not conclusively proved.

A number of problems remain unsolved. Techniques available for the detection of platelet agglutinins are, probably, inadequate to detect finer anti-

genic differences among platelets. It is, therefore, quite possible that many more platelet groups and types exist than have been recognized thus far. From the point of view of a convenient working hypothesis the distinction between groups and types is justified. It must be admitted, however, that the dividing line is probably a thin one. We have used "naturally" occurring agglutinins to determine platelet groups. It is true that they are found in healthy individuals who have never previously been transfused nor have been pregnant. There is, nevertheless, no certainty that these agglutinins have not developed in response to the stimulus of an unknown antigen. The use of "immune" plasmas for the detection of platelet types is also not free from criticism. In this technique, we use agglutinating plasmas from individuals who had been receiving repeated platelet transfusions. But obviously the group and the type of the platelets received by the patients may have greatly differed from donor to donor and the "immune" agglutinin we obtain is very likely directed against multiple antigens. For this reason, absorption studies in vitro and immunization studies in vivo become imperative and cause slow and painstaking progress. This difficulty may probably be overcome. Our attempts to develop platelet type agglutinins in group-compatible individuals have not been uniformly successful. Preliminary work, however, indicates that patients with thrombocytopenia may be better producers of "immune" plasma when injected with type-incompatible platelets. Of even greater value should be the plasma of nonthrombocytopenic mothers who deliver a thrombocytopenic child. In some cases this situation may be the consequence of platelet incompatibility between mother and fetus[7] and would supply "type-pure" agglutinin for platelet-typing purposes. At the present time, some of our findings may even be interpreted to deny the existence of individual platelet types. We have mentioned failure to produce immune agglutinins by injecting platelets of the compatible group. Moreover, two patients who had developed "type" immune agglutinins following repeated platelet transfusions responded poorly to the administration of platelets from donors, both "compatible" and "incompatible" by in vitro tests (Fig. 5). Their plasma caused significant thrombocytopenia in "compatible" as well as in "incompatible" recipients (Fig. 6). One would expect that these patients should respond favorably to the transfusion of compatible platelets and fail to induce thrombocytopenia in compatible recipients, should these effects be expression of platelet type-incompatibility. We are rather inclined to believe that these results indicate the extreme complexity of the antigenic structure of platelets.

Group and type differences in platelets have also important practical aspects. One has been indicated by Harrington and co-workers[7] with the description of a type of neonatal thrombocytopenia due to mother-fetus platelet incompatibility. Another is the all-significant problem as to whether the administration of group- and type-compatible platelets to thrombocytopenic patients may reduce the incidence of development of platelet agglutinins in patients receiving multiple platelet transfusions, a complication which threatens to nullify our efforts toward preservation and administration of platelets to some thrombocytopenic patients for hemostatic purposes. It remains to be estab-

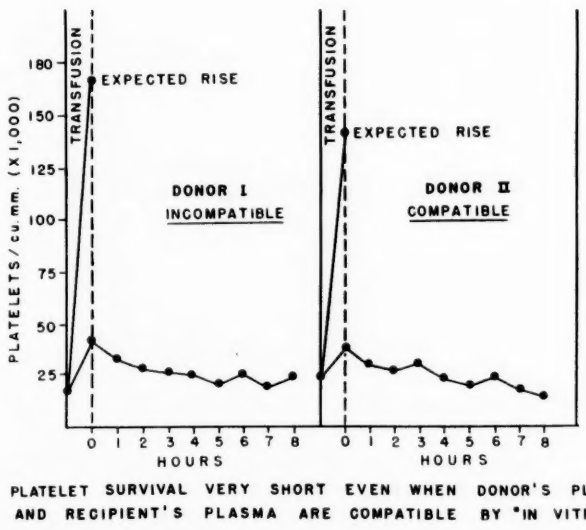

Fig. 5.—Survival of compatible and in incompatible type platelets in a patient of amegakaryocytic thrombocytopenia who had developed strong platelet agglutinin (1/16). (Note that platelets from both compatible and incompatible donors were quickly disposed of in the circulation, *even when compatibility was suggested by in vitro tests.*)

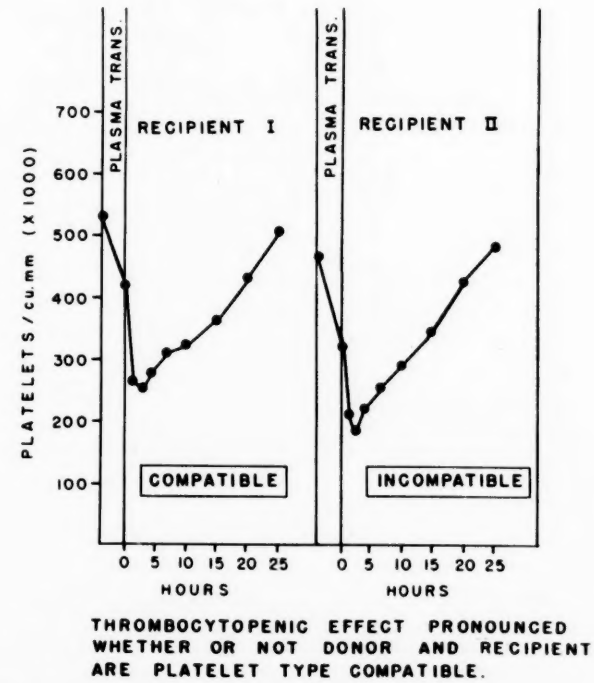

Fig. 6.—Thombocytopenic effect of platelet-immunized plasma when injected in compatible and incompatible recipients. Note that the thrombocytopenic effect was equally pronounced in all cases, *even when the plasma failed to agglutinate the recipient's platelets in vitro.*

lished whether the administration of group- and type-compatible platelets would materially reduce the occurrence of platelet isosensitization in thrombocytopenic patients.

Two additional findings in the course of this work require comment. One is the development of platelet *auto*agglutinins and thrombocytopenia in 2 individuals receiving multiple platelet transfusions. This finding may explain the rather frequent occurrence of platelet autoagglutinins in patients with idiopathic thrombocytopenic purpura, particularly of the "chronic variety." It is possible that these develop in response to antigenic stimulation by platelet products or platelets damaged by various agents (viruses, etc.). Another significant observation is the disappearance of the autoagglutinin in 5 cases of idiopathic thrombocytopenic purpura following splenectomy (in rough time relationship with elevation of the platelet count) and the persistence of platelet isoagglutinins. In recently published reports much attention has been given to the possible role of the spleen in the pathogenesis of the thrombocytopenia of idiopathic thrombocytopenic purpura and the mechanism of recovery following splenectomy.[5, 7, 8, 14] It is generally agreed that the spleen in idiopathic thrombocytopenic purpura may: (1) inhibit formation and delivery of platelets from the megakaryocytes; (2) trap and destroy circulating platelets, *after* these have been damaged by platelet antibodies or other agents; (3) produce platelet antibodies. The last reported observation, which confirms findings by Harrington and collaborators,[7] may emphasize the role of the spleen in the production and activity of platelet autoagglutinins.

SUMMARY

Strong evidence for the antigenicity of platelets is afforded by the development of specific agglutinins against heterologous platelets in animals, and of iso- and (occasionally) autoagglutinins in patients (especially with "amegakaryocytic" or secondary thrombocytopenic purpura) receiving multiple platelet transfusions. Development of platelet agglutinins is not prevented by the administration of ACTH or cortisone, and occurs more promptly when "viable," well-preserved platelets are given. The detection of platelet iso- and autoagglutinins in patients with idiopathic thrombocytopenic purpura (particularly of the "chronic variety") is also suggestive of different antigenic structure of platelets.

By direct cross-testing techniques it has been possible to determine the existence of two naturally occurring platelet agglutinins in human beings. Four groups probably exist as determined on the basis of the antigens found in platelets: I, II, III (I + II) and IV (O: no antigen). Their incidence has been determined in 285 individuals. Agglutinin absorption tests and in vivo immunization tests have confirmed the presence of such groups. They are unrelated, but immunologically have the same significance as the ABO antigenic system of the red blood cell, since they are detected by the use of naturally occurring agglutinins.

By the use of "immune" plasmas (plasma from patients who had received repeated platelet transfusions) it has been possible to distinguish 6 serologically different platelet types. The incidence of the various types has also been determined. By agglutinin absorption techniques and in vivo immunization studies, only 3 serologic types have been confirmed (1, 3, and 6). Since they are detected with "immune" plasma, platelet types correspond to the Rh-Hr system of the red blood cell. No antigens comparable to the M-N system of the red blood cell have been thus far detected in the platelets. Notwithstanding these results, some doubt must remain as to the existence of platelet types since, in in vivo experiments, patients with platelet agglutinins quickly dispose of injected type-compatible platelets, while their plasma causes thrombocytopenia when injected in type-compatible normal recipients. The relationship of group- and type-antigens remains uncertain.

Occasional patients receiving multiple transfusions develop agglutinins against their own platelets (autoagglutinins) as well as isoagglutinins and then become thrombocytopenic. It is possible that some such mechanism, with the development of platelet auto-sensitization, may result in idiopathic thrombocytopenic purpura.

In some cases of idiopathic thrombocytopenic purpura, a platelet autoagglutinin may be detected. In 5 of 9 cases the agglutinin disappeared following successful splenectomy, although the platelet isoagglutinin, also present, persisted after the operation. This finding may indicate that autoimmune thrombocytopenia is improved by splenectomy, even if isoagglutinin production continues, and suggests an additional possible mechanism to explain the favorable effect of splenectomy in many cases of the disease.

Acknowledgments.—The authors wish to express their appreciation to Dr. Alexander S. Wiener, who revised a preliminary manuscript and offered many helpful suggestions. They also wish to thank the many students of Tufts College Medical School and the Boston Dispensary School of Medical Technology, who volunteered for many of these studies.

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THE UPTAKE OF SUBCUTANEOUS AND INTRAMUSCULAR SILVER-COATED RADIOACTIVE GOLD COLLOIDS BY LYMPH NODES IN DOGS

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INTRODUCTION

RADIOACTIVE gold colloids have seen increasing experimental and clinical use in recent years.[1] The pelvic lymphatic drainage of these sols has been investigated by Sherman and co-workers.[2] Meneely and associates[3] have reported on the distribution, survival, and pathology following transbronchial instillation in the dog. In the meantime, silver colloids and silver-coated gold colloids have been shown to be more efficiently drained by pulmonary lymphatics than their plain gold counterparts.[4-6] It was the purpose of this investigation to determine the efficiency of uptake by the regional lymph nodes of measured tracer quantities of silver-coated radiogold colloids from subcutaneous sites. It was felt that delineation of the rate of time lapse to this drainage process would be of interest. In addition, it seemed desirable to correlate the deep and superficial drainage of tracer quantities of colloid with the microscopic changes in regional nodes following the infiltration of activities approximating therapeutic quantities of silver-coated gold sols in the mammary area.

MATERIALS AND METHODS

The preparation of the silver-coated gold colloids has been described elsewhere.[6] Dogs 1 to 4 were injected subcutaneously at each site with 0.5 ml. of the colloid suspended in 6 per cent gelatin. The lower extremities were injected on the medial aspect of the thigh, approximately 2 inches proximal to the femoral condyle. For breast injections a site was chosen 2½ inches from the midline and 4 inches from the midpoint of the axilla. The sol was introduced into the left upper extremity on its medial aspect 1 inch proximal to the humeral condyle. Dogs 5 and 6 were injected in similar manner except that, in the latter, 150 T.R. units of hyaluronidase* were injected with the colloid, while in the former an equal volume (0.5 ml.) of normal saline was introduced with the sol. Dogs 1 to 6 were injected forty-eight, thirty-six, twenty-four, and twelve hours before being sacrificed. It has previously been noted that considerable drainage of silver-coated gold colloids introduced by bronchoscope may occur in two days,[6] so forty-eight hours was selected as an appropriate experimental sacrifice time. The sites were rotated to equalize any regional drainage differences. After autopsy the inguinal and axillary nodes were weighed. The injection sites were excised widely and included skin, subcutaneous tissue, fascia, and muscle. Since the weights of the tissues taken from these sites showed great variability, the tissues were put into pseudo solution in toto, using a mixture of concentrated nitric

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This study was carried out under Contract At-(40-1)-269 with the Division of Biology and Medicine, United States Atomic Energy Commission.
Received for publication, July 24, 1953.

and sulfuric acids. The resultant solutions were diluted and aliquots taken for counting. An aliquot of 1 ml. of the colloid as administered was saved for subsequent radioactivity measurements.

Animals 7 to 12 were injected in the left mammary region only at a point 2½ inches from the midline and 4 inches from the midpoint of the axilla. Dogs 7 and 8 received tracer quantities subcutaneously and intramuscularly, respectively. At autopsy five days later, tissue, indicated in Table III, was collected and assayed for activity. Animals 9 to 12 received large quantities of the colloid (45 mc. each). The latter pair received subcutaneous infiltrations, while the former pair were injected in the deep musculature of the chest wall. At autopsy twenty-one days later, axillary, retrosternal, supraclavicular, and inguinal nodes along with breast tissue were collected bilaterally. Sections of the liver and spleen were also obtained.

RESULTS

The data in Table I (Dogs 1 to 6) show that there is no outstanding or predictable difference between the node activities at the end of two days as compared with twelve-hour draining period by the methods used here. When the

TABLE I. PER CENT PER GRAM FRESH TISSUE OF TOTAL ACTIVITY FOUND IN REGIONAL LYMPH NODE AT TWELVE, TWENTY-FOUR, THIRTY-SIX, AND FORTY-EIGHT HOURS

DOG NO.	12 HR.	24 HR.	36 HR.	48 HR.	
1	0.5	2.3	1.8	0.1	
2	2.3	2.8	1.5	7.4	
3	0.9	1.0	1.6	1.3	
4	0.9	1.5	1.6	.008	
5	0.9	1.5	1.5	2.4	N Saline
6	4.7	2.2	7.0	4.6	Hyaluronidase
Average values	1.7	1.9	2.5	2.6	

TABLE II. PER CENT OF TOTAL ACTIVITY RECOVERED FROM INFILTRATION SITE AFTER INDICATED TIME PERIODS

DOG NO.	12 HR.	24 HR.	36 HR.	48 HR.	
1	88.5	76.8	62.4	96.7	
2	3.4	54.6	55.4	23.3	
3	56.8	68.6	56.6	19.5	
4	46.4	60.2	68.1	50.4	
5	78.0	82.6	77.6	81.0	N Saline
6	74.8	60.6	90.6	75.3	Hyaluronidase
Average values	59.9	67.2	68.4	57.7	

TABLE III. LYMPHATIC DISTRIBUTION OF SILVER-COATED GOLD COLLOID FOLLOWING SUBCUTANEOUS AND INTRAMUSCULAR INFILTRATION OF TRACER QUANTITIES

DOG	TOTAL COUNTS/ MIN. INFIL.	RT. AX. NODE*	L. AX. NODE*	L. RETRO. STERNAL NODE*	RT. RETRO. STERNAL NODE*	L. MED. NODE*	SKIN OVER RT. BREAST*	TOTAL ACTIV. IN LIVER*	LIVER ACTIV. PER GM. FRESH TISSUE	% TOTAL ACTIV. FOUND IN LIVER	ROUTE
7	3,000,000	205	53,800	6,740	2,225	97	192	21,984	48	0.70	S.C.
8	3,000,000	0.0	3,100	40,000	20,000	254	0.0	52,600	116	1.75	I.M.

*Counts per minute per gram fresh tissue.
±Drainage time both dogs five days.

*Obtained through courtesy of Wyeth, Inc.

results from individual dogs are averaged, there appears a steady increase in nodal activity through the forty-eight hour period. The use of hyaluronidase in conjunction with the colloid resulted in higher levels of activity in the nodes for a comparable time period. It will also be noted that the thirty-six hour nodes displayed the most consistent levels of activity.

Table II (Dogs 1 to 6) illustrates that removal of colloid from the infiltration site was fairly efficient, although aberrant values are noted in Table II for Dogs 2 and 3. The data do not permit any clear-cut prediction to be made concerning the quantity of colloid that would be removed with passage of a definite time period. Here again the differences between activities for twelve and forty-eight hour sites are not consistent.

The site of the node seemed to exert relatively little influence on the amount of subsequent activity recovered.

The mean activity for all nodes (Dogs 1 to 6) regardless of location and drainage age was calculated to be 2.3 per cent of the total infiltrated activity per gram fresh tissue, while the median value was 1.6 per cent.

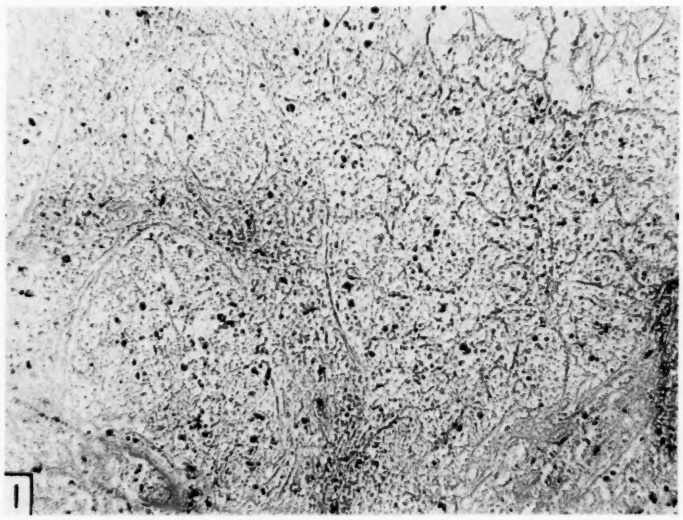
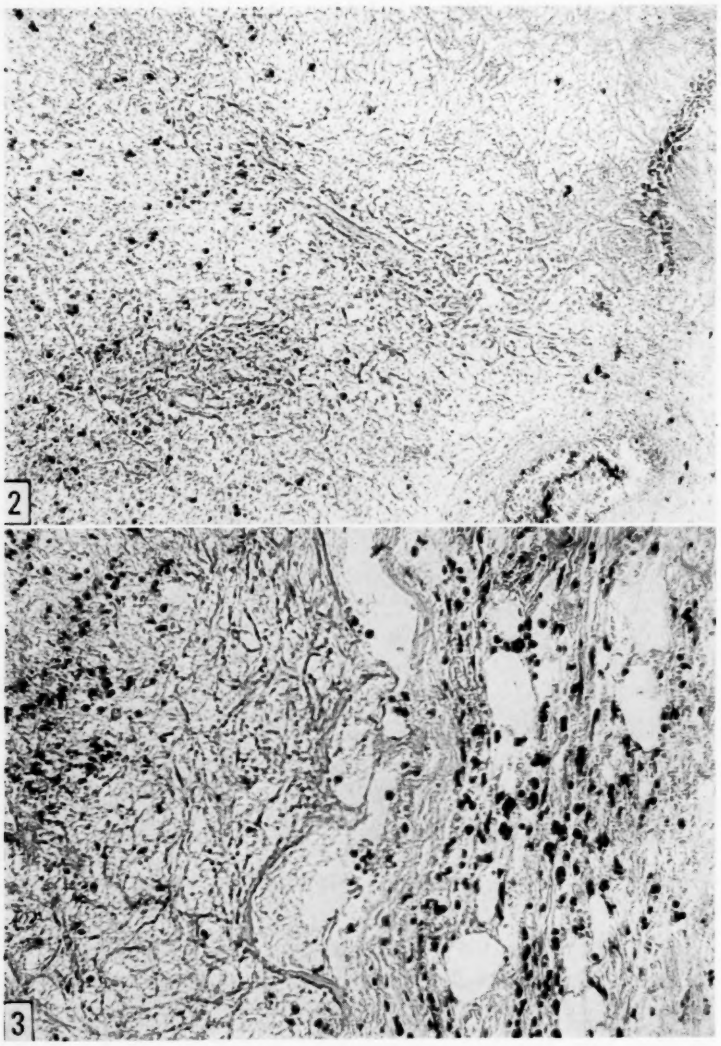


Fig. 1.—The left retrosternal node of Dog 10 is highly atrophic with great decrease in cellular elements. Reticular proliferation is slight. (I.M. Infiltration 45 mc.) (×150.)

Table III (Dogs 7 and 8) represents an attempt to quantitate the difference between deep and superficial drainage in the mammary region over a five-day period. The left axillary node displays 17 times more activity in the superficially injected Dog 7. The retrosternal nodes are approximately 6 times as active in Dog 8 as in Dog 7. Other deep tissue as expected shows greater activity in Dog 8. The contralateral retrosternal nodes display about 50 per cent of the activity of homolateral nodes.

When larger quantities of gold were employed (Dogs 9 to 12, 45 mc. each), the same general trend is noted in so far as differences in node activities are concerned, as in Dogs 7 and 8. The microscopic changes in deep and super-

ficial nodes of Animals 9 to 12 are consistent with what one would predict from the data obtained using tracer quantities in Dogs 7 and 8. For example, the left retrosternal nodes of Dogs 9 and 10 were highly atrophic (Fig. 1) while the left axillary nodes were near normal in appearance. Conversely, the left axillary nodes were highly atrophic in Dogs 11 and 12 (Figs. 2 and 3)



Figs. 2 and 3.—The left axillary nodes of Dogs 11 and 12 show great destruction of lymphoid elements. There is some reticular proliferation. (S.C. Infiltration 45 mc.) (×150.)

while the retrosternals for both dogs showed only slight changes (Fig. 4), notwithstanding the high activity found in the retrosternals of the latter two animals. The supraclavicular nodes from these four animals were near normal in appearance, with some hemosiderin seen in scattered areas.

DISCUSSION

In previous publications[5, 6] it was pointed out that the lymphatic drainage of silver-coated gold colloids is more efficient than straight gold colloids over a comparable time period. The fundamental mechanism operating to effect this difference in drainage ratio is not understood.

The mean node uptake of 2.3 per cent per gram fresh tissue here seems insignificant in comparison with the total infiltrated activity, but when the small size of the node is considered the result in equivalent roentgen dose delivered is considerable.[6] The data represented by Animals 1 to 6 would indicate that sufficient colloid is deposited in the regional lymphatics in twelve to forty-eight hours to achieve destructive levels when larger quantities are employed. As would be expected, the concomitant use of hyaluronidase seems to enhance the drainage. In addition, it is possible that more activity traverses the nodal tissue than can be recovered therein at any one time. The work of Bangham and co-workers,[7] however, would tend to dispute loss from the nodes per se.

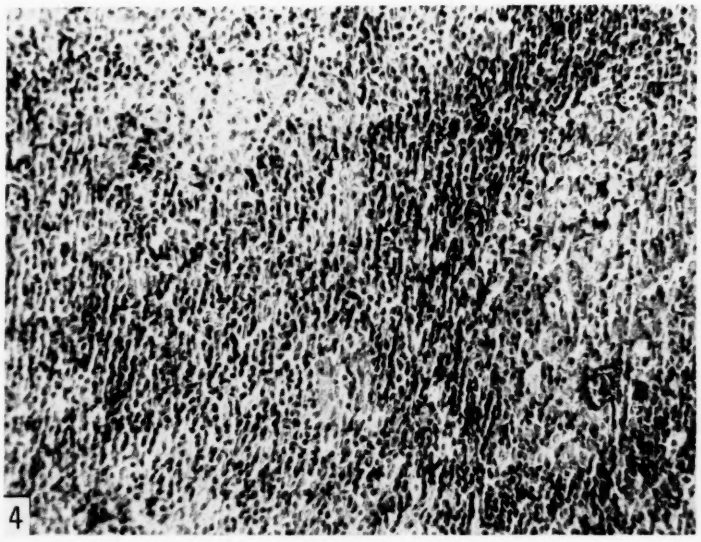


Fig. 4.—The left retrosternal node of Dog 12 displays no congestion or degeneration. The capsule of this node was thickened, otherwise it was normal in appearance. (S.C. Infiltration 45 mc.) (×150.)

The results obtained from Animals 7 and 8 give a crude quantitative difference in drainage of silver-coated gold colloids by contra- and homolateral axillary and retrosternal nodes from superficial and deep sites on the chest wall.

The data presented in Tables I and III would indicate the desirability of using large doses of silver-coated gold colloids in experimental animals with subsequent radioactivity measurements and microscopic sections of draining

nodal tissue. In this regard, nodal destruction when 45 mc. of the sol are used is apparent in Figs. 1, 2, and 3. The axillary nodes of the superficially injected dogs show destruction throughout 90 per cent of their substance, but the remaining portion seems well preserved though congested. Whether this process had gone to completion in the 21-day postinjection period is to be considered, or it may be that communicating channels within the node were disrupted, making the remaining 10 per cent of well-preserved tissue inaccessible to the gold particles. The hyalinized nodes displayed greatest destruction centrally. The microscopic changes in the left retrosternal nodes of the intramuscularly injected dogs (Fig. 1) showed atrophic changes throughout. On the other hand, the contralateral retrosternal nodes which are located approximately 0.5 cm. from the homolateral nodes in the dog showed only congestion with no atrophic changes (Fig. 5). To destroy both retrosternals it may be necessary to make deep injections on both sides of the midline or a least a larger single injection closer to the midline. Whether or not this method of injection would be more efficient than an intrapleural injection in eradicating these nodes is open to question. Destruction of internal mammary nodes could possibly be accomplished by deep injections at the various interspaces.

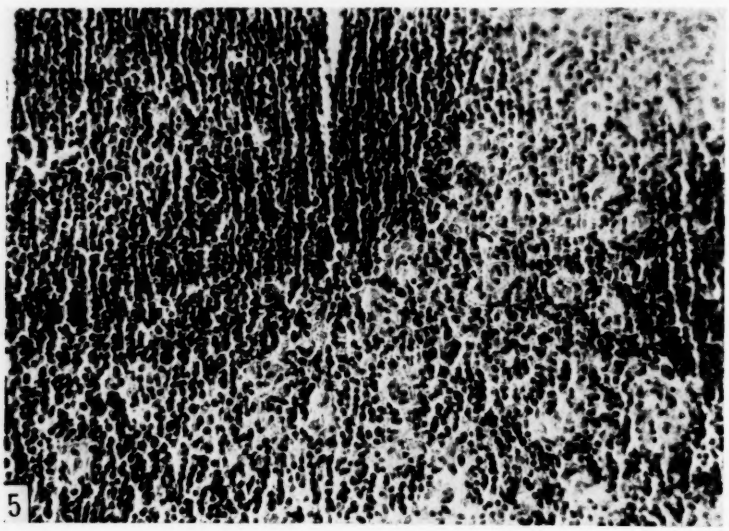


Fig. 5.—The lymphoid element of this node of Dog 9 appears intact. The node was, however, slightly congested. (I.M. Infiltration 45 mc.) (×150.)

Though some of the infiltrated activity can later by recovered from the liver (Table III), the amount of radiation derived from such a route of administration would appear to be well tolerated by hepatic tissue.[8]

Possibly some use could be found for these colloids where it is desirable to destroy inaccessible nodes or to prevent the organization therein of autonomous cells using subcutaneous or intramuscular routes of administration.

SUMMARY

Silver-coated radioactive gold colloids have been administered to dogs by the subcutaneous route and have been shown to be taken up by the regional lymph nodes in significant quantities. The quantitative differences between drainage from superficial and deep breast sites were noted. Some microscopic changes in regional nodes following infiltration of a large single dose of silver-coated gold colloid were presented and discussed.

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THE EFFECT OF LARGE DOSES OF CORTISONE UPON THE ABILITY OF THE RETICULOENDOTHELIAL CELLS TO PHAGOCYTOSE STREPTOCOCCI

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IT HAS been demonstrated experimentally by the occurrence of spontaneous lesions in the visceral organs that susceptibility to infection is greatly increased in mice, rats, and rabbits by the administration of large doses of cortisone.[1-5] The cause of this increase of susceptibility has been attributed to a suppression of antibody development[1] and to suppression of the activity of circulating leukocytes in the blood.[6] Teilum, Engberk, and Simonsen[7] have found an anatomically altered condition in the reticuloendothelial cells in animals treated with cortisone. These cells are known to be actively phagocytic for bacteria in the blood stream and to be the chief cellular defense in a bacteremia. We have attempted in the following experiments to find if the ability of these cells to phagocytose may be altered in rats in any way by giving large doses of cortisone and if this alteration might be a factor in the increase of susceptibility to infection.

MATERIALS AND METHODS

The white rat was used. In most cases young rats weighing about 50 grams were selected. In all rats a dose of cortisone consisting of 1 mg. per 10 grams of animal was given subcutaneously daily for seven days. On the eighth day 0.5 ml. of a twenty-four hour broth culture of *Str. viridans* was injected into the tail vein of both treated and normal animals. Animals were killed after the intravenous streptococcic injection at intervals of fifteen minutes, one hour, six hours, eight hours, twenty-four hours, forty-eight hours, seventy-two hours, and ninty-six hours. Small pieces of liver were quickly removed from the left lobe of the liver, fixed in 10 per cent formaldehyde, and stained by the Gram method. The ocular of the microscope was fitted with a micrometer which divided the field into quadrants to make it possible to count the bacteria in the Kupffer's cells more accurately. In each animal the average number of bacteria per quadrant in 25 different quadrants, the average number of bacteria per each of the Kupffer's cells which exhibited phagocytosis and the percentage of the 25 fields which showed bacteria were observed and determined.

THE RESULTS OF THE EXPERIMENTS

The averages of the findings of 25 fields in each animal are tabulated in Table I. The bacteria per field were slightly greater in the treated animals at the earliest determination, fifteen minuts, and continued to remain greater until between twenty-four and forty-eight hours when all of the organisms had disappeared from the normal animals. The number of bacteria per positive phagocytic cell was also greater in the treated animals, but there was less variation. These bacteria were also completely destroyed in the normal animals between twenty-four and forty-eight hours after the intravenous injection of streptococci.

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This study was aided by a grant from the United States Public Health Service.
Received for publication, July 7, 1953.

TABLE I. EFFICIENCY OF THE KUPFFER'S CELLS IN CORTISONE TREATED AND NORMAL RATS TO PHAGOCYTOSE STREPTOCOCCI AT VARIOUS INTERVALS AFTER THE ANIMALS HAD BEEN INJECTED INTRAVENOUSLY

THE AVERAGES OF THE FINDINGS IN 25 FIELDS IN EACH ANIMAL								
CORTISONE TREATED ANIMALS					NORMAL ANIMALS			
NO. OF ANIMALS	TIME	BACTERIA PER FIELD	BACTERIA PER POSITIVE CELLS	PER CENT OF POSITIVE FIELDS	NO. OF ANIMALS	BACTERIA PER FIELD	BACTERIA PER POSITIVE CELLS	PER CENT OF POSITIVE FIELDS
10	15 min.	6.6	5.2	72.0	10	4.6	4.0	68.8
4	1 hr.	6.2	5.5	73.0	4	3.8	4.6	56.0
4	6 hr.	4.5	4.2	59.0	5	3.1	4.6	30.0
5	8 hr.	6.0	4.4	71.0	5	4.0	4.1	54.4
4	24 hr.	1.6	4.0	28.0	5	0.3	3.2	8.8
7	48 hr.	0.6	3.3	15.4	5	0.0	0.0	0.0
5	72 hr.	0.08	1.6	0.8				
4	96 hr.	0.0	0.0	0.0				

The percentage of positive fields decreased in both groups of animals to where it was 15.4 per cent in the treated animals and 0.0 per cent in normal animals at between twenty-four and forty-eight hours. The organisms in the treated animals were not completely destroyed until between seventy-two and ninety-six hours after the intravenous injection.

DISCUSSION

While it has been conclusively shown that susceptibility to infection in mice, rats, and rabbits is greatly increased by the administration of large doses of cortisone,[1-5] the mechanism of bringing about the increase is not completely understood. It is the opinion of Antopol[1] that the frequency of infection is due to cortisone which depresses antibodies and permits a multiplication in the body of dormant pathogenic or ordinary nonpathogenic organisms. Antibodies have been found not to be changed by some observers.[8-11] Others have found antibodies reduced.[12-15] We[4, 16] observed no difference in the concentration of streptococcic antibodies in animals treated with cortisone and in normal animals. It seems that it is doubtful whether the reduction of antibodies circulating in the blood stream is an entirely satisfactory explanation of the rapid increased susceptibility to infection with heavy cortisone treatment.

According to Antopol,[1] the virulence of organisms in mice does not appear to be increased. Our experiments have shown that organisms isolated from abscesses in rats when cultured and injected intraperitoneally in large doses have no ill effect upon normal rats. It would seem more reasonable to believe that some change takes place in the host which causes increased susceptibility to infection.

Crepea and associates[6] reported a reduction in the ability of the leukocytes in the blood to phagocytose in 9 of 10 patients treated with cortisone. Mogabgab and Thomas[5] observed no difference in the ability of leukocytes to phagocytose in normal rabbits and in rabbits treated with cortisone.

It appears that the Kupffer's cells and probably the reticuloendothelial cells in other locations in animals heavily treated with cortisone, while engulfing organisms fully as efficiently if not better than cells in normal animals,

are depressed in the ability to destroy organisms. One might speculate and assume that organisms are held and permitted to multiply and produce lesions (abscesses). So far in our experiments the only factor found which could definitely contribute to the increased susceptibility to infection associated with large doses of cortisone is the reduced ability of the reticuloendothelial cells to destroy organisms which had been engulfed. In animals treated with smaller doses of cortisone (1 mg. per 500 grams of animal) the ability to phagocytose is not changed.

SUMMARY

The efficiency of the reticuloendothelial cells (Kupffer's cells) to engulf bacteria appears to be about equal in the rats treated with large doses of cortisone and in normal rats, but the ability of these cells in the treated animals to destroy the engulfed organisms is definitely depressed. This depression of the reticuloendothelial cells may be an important factor in the increased susceptibility to infection in animals treated with large doses of cortisone.

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LABORATORY METHODS

METHODS OF ESTIMATION OF ADRENAL CORTICAL STEROIDS WITH TETRAZOLIUM SALTS

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SINCE the isolation and identification of the adrenal cortical steroids, a number of chemical methods for their quantitation have been introduced. The methods commonly used are based on the fact that all the known biologically active cortical steroids possess an alpha ketol (acyloin) grouping at the C_{20} and C_{21} positions.[1, 2, 3] One exception is the periodic acid oxidation method which is applicable to cortical steroids having either the alpha ketol or alpha diol grouping at C_{20} and C_{21}.[4]

Each of the methods of color development employed for steroid estimation has its limitations. These relate to the sensitivity and specificity of the reagents as well as to their simplicity and stability. Preliminary observations indicated that use of tetrazolium salt as an oxidizing agent in steroid estimation might be an improvement over the agents usually employed for this purpose.

METHODS AND MATERIALS

Early observations indicated that triphenyl tetrazolium chloride gave transient color with corticosteroids in alkaline solution while 3,3′-dianisole-bis-4,4′-(3,4-diphenyl)-tetrazolium chloride (BT or blue tetrazolium)[5] was found to yield a stable product when reduced by active corticosteroid. This compound is reduced rapidly in alkaline solutions to give a bluish-red coloration by all five of the cortical hormones tested. The color produced follows closely Beer's law.

When completely reduced, blue tetrazolium gives a pure, dark blue pigment, 3,3′-dianisole-bis-4,4′(3,5-diphenyl) formazan. However, when half of the molecule is reduced, the pigment produced is red.[5] Assuming that both moieties of the molecule are reduced at approximately the same rate, the large excess of the reagent to the steroids should give mostly semireduced tetrazolium salt. It is predominately this semireduced product plus some totally reduced compound that one measures.

A number of other tetrazolium salts were tested including 2,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride (Iodo RT).[6] The reduced form of the latter compound gave a pure shade of red. This color is stable over many hours. However, since it was unavailable commercially and since its sensitivity was less than that of tetrazolium blue, it was not investigated extensively.

The absorption maxima of both BT and Iodo RT tetrazolium shown in Fig. 1 occur at nearly the same wavelength of light. The greater color development of the blue tetrazolium in the presence of DOC as compared to RT is shown in Fig. 2.

Though complete solution in methanol does not designate its purity, suitable blue tetrazolium must be freely soluble in methanol at 1 per cent concentration. The melting

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Supported in part by a United States Public Health Service Grant.
Received for publication, June 2, 1953.

point (decomposition point) of the compound is not sharp and cannot be depended upon as a good criterion of its purity. One non-oxidizing substance found in more than 50 per cent quantity in an impure sample of blue tetrazolium melted and decomposed at a similar temperature to blue tetrazolium. In practice, a few presumably pure batches of tetrazolium salt may be tested against desoxycorticosterone. The batch that gives the highest reading is saved as a standard and the other batches are digested in boiling water and repeatedly recrystallized from a methanol and acetone mixture or a methanol and ethyl acetate mixture until color production with a given amount of desoxycorticosterone becomes maximal. It was found to be more satisfactory to synthesize[5] tetrazolium than to purify the commercial product.

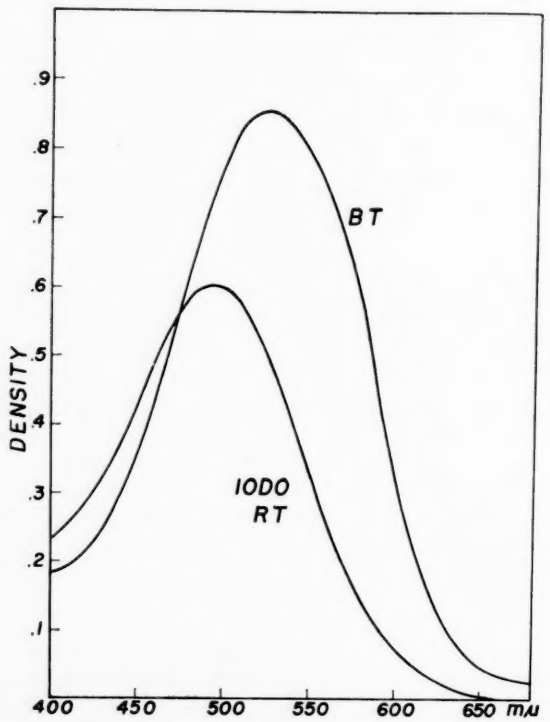

Fig. 1.—Optical density curve of blue tetrazolium and of iodo-red tetrazolium reduced with 50 micrograms of desoxycorticosterone and choline.

Studies were undertaken to determine the optimum conditions for color development with BT in the presence of reducing steroids. It was found that both the alkali and solvents used influenced the extent, rate of color formation, and its stability. The rate of color development of various reducing steroids with sodium hydroxide or choline is shown in Figs. 3 and 4, respectively. It is apparent that the rate of color development is more rapid in sodium hydroxide. However, the slopes of the curves in Figs. 5 and 6 demonstrate the greater sensitivity of the choline procedure as compared to that employing sodium hydroxide. Reduction by the relatively inactive steroid hormones was essentially the same in both instances.

Other organic bases such as benzyl-trimethylammonium hydroxide (Triton B), tetramethylammonium hydroxide, and tetraethylammonium hydroxide, also proved to be satisfactory sources of base. The solvent of choice when using organic bases is ethanol, the purity of which is not critical. Redistilled absolute or 95 per cent ethyl alcohol may be used. The purity of the methanol was found to be of the greatest importance for the success of the

determination with sodium hydroxide as an alkali. Occasionally a batch of methanol is so completely unsuitable that even repeated distillation with dinitrophenylhydrazine and sulfuric acid, solid sodium hydroxide, and zinc with hydrochloric acid will not remove whatever interfering substance or substances are present. When sodium hydroxide is used as an alkali, pyridine:hydrochloric acid is needed to adjust the solution to the desired pH at which the color formed is stable for many hours, whereas the color is stable without this step if an organic base is used as a source of alkali.

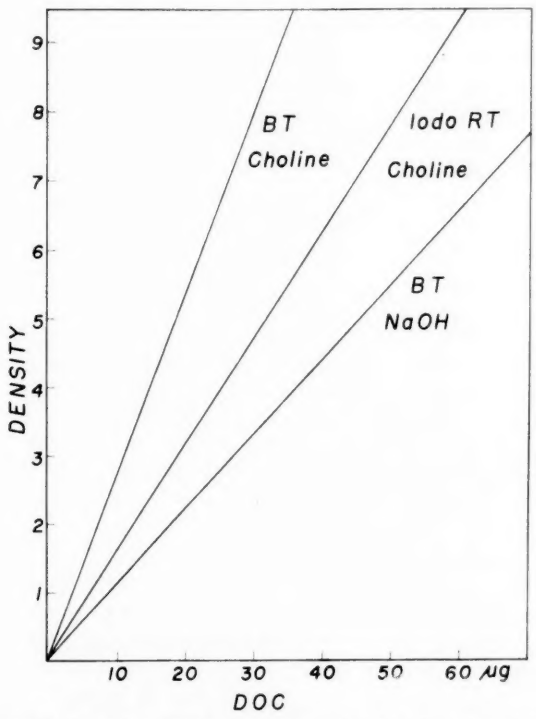

Fig. 2.—Comparison of the color development by reduction of tetrazolium dyes with desoxycorticosterone.

The extent of color development of various steroids in the presence of BT and using choline as a base is shown in Fig. 6. It is apparent from the sharp division between compounds with a reducing side chain and those without, that the reduction of BT is largely dependent upon the side chain and that variations in ring structure have but little effect on the reduction of tetrazolium. The spread in color development between compounds is less when the results are expressed on a molar basis.

The blanks are lower and the sensitivity higher when the quantities of the reagents are kept small and the reaction is carried out in the absence of light.

Two procedures are presented in this paper, one using sodium hydroxide and the other using choline as the base, both with blue tetrazolium.

Procedures.—

A. *Blue tetrazolium:Sodium hydroxide procedure:*
Reagents:

Absolute methanol: Reagent grade, Mallinckrodt, distilled from dinitrophenylhydrazine (10 Gm. per liter). The fraction distilled between 63.5° C. and 65.0° C. is collected.

Blue tetrazolium (BT)*: Digested with boiling water and recrystallized from methanol and acetone mixture. M.P. 242-245° C. dec.

1 per cent methanolic solution: 1 gram blue tetrazolium is dissolved in methanol and diluted to 100 ml. The solution is prepared the day it is to be used and is kept in an amber glass-stoppered bottle.

Pyridine: Dried over barium oxide and distilled in vacuum; only the middle fraction of the distillate is saved.

Acid-Pyridine: 20 ml. of concentrated hydrochloric acid is added gradually to 100 ml. of freshly distilled pyridine.

Methanol-Acid-Pyridine: 3 parts of methanol are mixed with 1 part of acid-pyridine the day this reagent is to be used.

1.0N Sodium hydroxide in 80 per cent methanol: 40 Gm. of reagent grade hydroxide pellets are dissolved in 200 ml. double distilled water. The solution is cooled and diluted to 1,000 ml. with redistilled absolute methanol, mixed thoroughly, titrated against standard acid and adjusted to exactly 1.0N.

Colorimetric Determination.—1 ml. of methanolic solution of steroid or steroid extract, 1 ml. methanolic sodium hydroxide, and 1 ml. blue tetrazolium are mixed in a glass-stoppered test tube and allowed to stand in the dark in a water bath at 25° C. for exactly twenty minutes. The reaction is stopped at this point by the addition of 4 ml. methanol-acid-pyridine mixture and is mixed thoroughly. It is read at 515 mμ, filter PC-4, in a Coleman Model 14 spectrophotometer or at filter 515, 6.0 aperture, in an Evelyn colorimeter.

The color is stable for about one hour without the addition of the methanol-acid-pyridine buffer and is stable for a few hours with the buffer.

B. *Blue tetrazolium:Choline procedure.*—

Reagents:

Ethanol: Absolute or 95 per cent ethanol is distilled from dinitrophenylhydrazine and sulfuric acid, or from blue tetrazolium and alkali; the middle fraction is taken.

Blue tetrazolium (BT) solution: The 0.5 per cent ethanolic solution is prepared by dissolving 500 mg. blue tetrazolium in 100 ml. distilled ethanol in a volumetric flask. The solution is stable for at least one week when kept in a refrigerator.

Choline solution: 2 ml. 50 per cent aqueous choline solution (Rohm and Haas) is diluted to 100 ml. with distilled ethanol. The solution is stable for at least a week when kept in a refrigerator.

Colorimetric Determination.—1.8 ml. ethanolic solution of steroid or steroid extract, 0.1 ml. choline solution and 0.1 ml.† blue tetrazolium solution are mixed well in a glass-stoppered test tube and allowed to stand in the dark at room temperature (26 to 28.5° C.) for two hours. Five milliliters of distilled ethanol is added and the solution is read in a colorimeter at 515 mμ, as described in "A."

*Commercial samples were obtained from Dajac Laboratories Division, Monomer-Polymer, Inc., Leominster, Mass., and General Biochemicals, Inc., Chagrin Falls, Ohio.

†All the results using organic bases presented in this paper are obtained by following the procedures given. The minimum quantities of reagents are recommended. For the determination or larger quantities of steroids, it is advisable to use more BT; there is little difference in color production in using 0.1 to 0.8 ml. of 0.5 per cent BT. However, though larger quantities of choline will shorten the reaction time, high blanks will result.

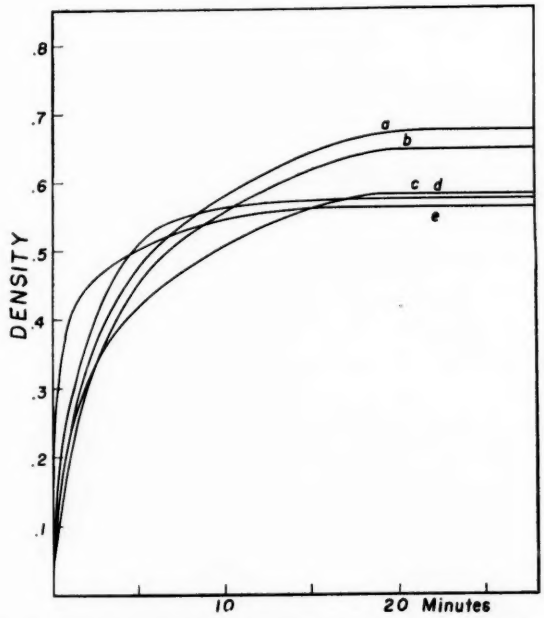

Fig. 3.—Rate of reaction by the BT:NaOH procedure of 0.303 micromole of the following steroids: *a*, Desoxycorticosterone; *b*, Corticosterone; *c*, 17 α-hydroxy-desoxycorticosterone; *d*, 17 α-hydroxy-corticosterone; *e*, 17 α-hydroxy-11-dehydrocorticosterone.

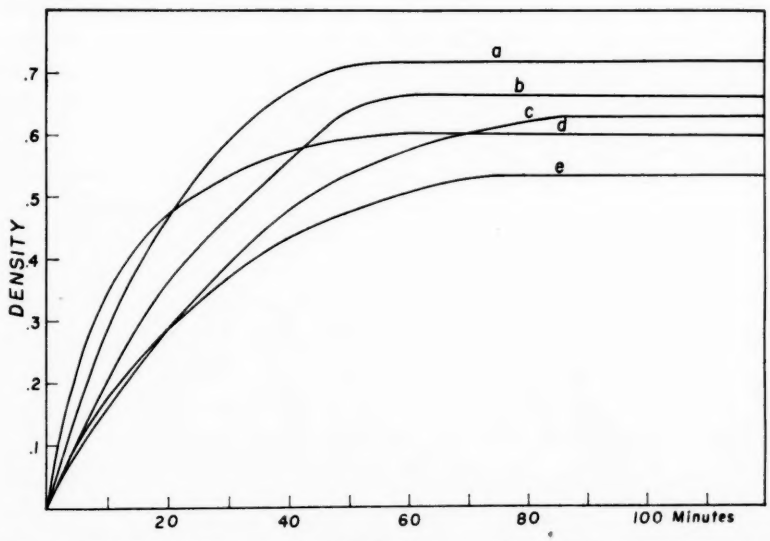

Fig. 4.—Rate of reaction by the BT:Choline procedure of 25 micrograms of the following steroids: *a*, Desoxycorticosterone; *b*, Corticosterone; *c*, 17 α-hydroxy-desoxycorticosterone; *d*, 17 α-hydroxy-11-dehydrocorticosterone; *e*, 17 α-hydroxy-corticosterone.

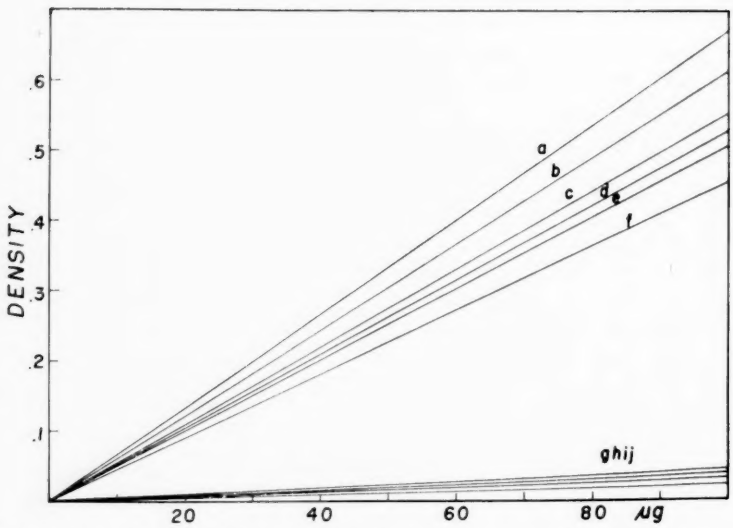

Fig. 5.—Standard curves of optical density versus concentration for the following steroids using the BT:NaOH procedure: *a*, Desoxycorticosterone; *b*, Corticosterone; *c*, 17 α-hydroxy-11-desoxycorticosterone; *d*, 17 α-hydroxy-corticosterone; *e*, 17 α-hydroxy-11-dehydrocorticosterone; *f*, 17 α-hydroxy-11-dehydrocorticosterone acetate; *g*, 17 α-hydroxy-progesterone; *h*, Progesterone; *i*, Cholestenone; *j*, 7-keto-cholesteryl acetate.

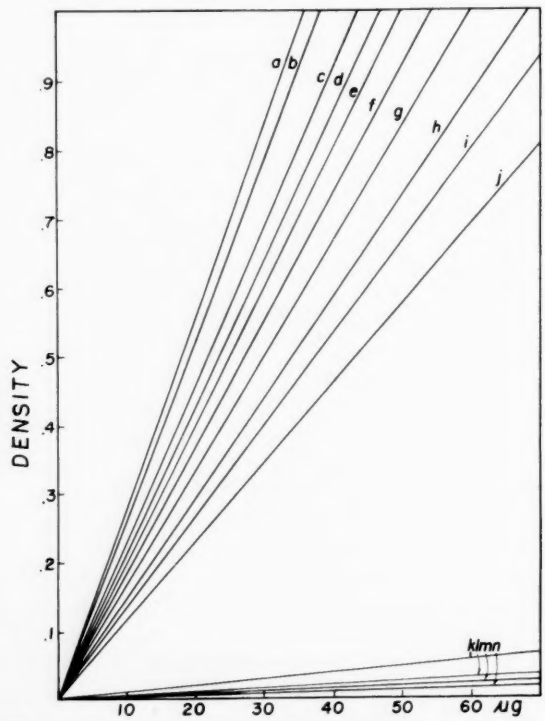

Fig. 6.—Standard curves of optical density versus concentration for the following steroids using the BT:Choline procedure: *a*, Desoxycorticosterone; *b*, Corticosterone, Desoxycorticosterone acetate; *c*, 17 α-hydroxy-11-desoxycorticosterone; *d*, 17 α-hydroxy-11-dehydrocorticosterone; *e*, 17 α-hydroxycorticosterone; *f*, 17 α-hydroxy-11-dehydrocorticosterone acetate; *g*, 17 α, 21-dihydroxy-pregnane-3,11,20-trione acetate; *h*, 3 α, 17 α, 21-trihydroxy-pregnane-11,20-dione acetate; *i*, 17 α, 20-dihydroxy-pregnane-3,20-dione acetate; *j*, Δ 5-pregnene-3 β, 17 α, 21-trihydroxy-20-one diacetate; *k*, 17 α-hydroxy-progesterone; *l*, Progesterone; *m*, Cholestenone; *n*, 7-keto-cholesteryl acetate.

DISCUSSION

The reducing power of the steroids is due chiefly to the acyloin group. The 4-3 ketones such as cholestenone, testosterone, and progesterone also exert some reducing power (Figs. 5 and 6). The quantities of these steroids necessary to produce the same amount of coloration as the corticosteroids are so much larger that they are unlikely to interfere greatly with the accurary of the results when urine or blood is analyzed for corticoids. Dextrose, ascorbic acid, cysteine, glucuronic acid, unsaturated fatty acids and their derivatives, and other reducing compounds all cause color formation of the tetrazolium salt and thus interfere with the results when present. However, with the exception of the fatty acid derivatives, they are all easily soluble in water and less soluble in organic solvents and thus will not pose any problem in the determination. Suitable methods of separation must be adopted to be rid of unsaturated fatty acids, phospholipids, and glycerides before an estimation is made. The same compounds also interfere with the copper reduction, phosphomolybdate reduction, and the periodic acid oxidation methods.

Earlier it was reported[7] that 5 micrograms of any of five cortical hormones could be measured accurately when 1N sodium hydroxide and BT in methanol were used. More recently, Mader and Buck[8] reported a similar procedure employing tetramethylammonium hydroxide as the alkali. Their procedure gives higher blank readings than are obtained in the BT:choline procedure described in this paper. Their method gave similar results when the quantities of reagents were reduced. However, alcoholic tetramethylammonium hydroxide is less stable than the solution of choline.

In the meantime, two papers have been published by Henly[9, 10] using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride and sodium hydroxide in pyridine. This procedure was reported to be slightly more sensitive than the BT:NaOH method described here.

Sodium hydroxide has the advantage over the organic bases of being a common reagent in every laboratory. The color is stable for hours after the addition of acid:pyridine buffer. The color by the three procedures using organic bases is likewise stable over many hours.

Esters such as desoxycorticosterone acetate may also be determined in the same manner. Some esters require a longer time of reaction in certain procedures due probably to slow hydrolysis at the particular alkaline strength.

A comparative study of urinary corticosteroids by the two procedures favored in this laboratory, BT:choline and BT:NaOH shows good agreement (Table I).

Alpha, beta-unsaturated ketones produce some color, as do alpha ketoles in positions other than C_{20} and C_{21} (Figs. 5 and 6). The time for complete reaction is longer and the color formed by equivalent amount is too faint ($\frac{1}{10}$ to $\frac{1}{20}$ molar equivalent) to interfere greatly with the steroid determination. The same reducing groupings will similarly interfere with copper reducing

TABLE I. COMPARISON OF BLUE TETRAZOLIUM METHODS FOR REDUCING STEROID ESTIMATION ON THE URINE OF HUMAN SUBJECTS*

	BT:NaOH	BT:CHOLINE
Range	6.5-64	7.0-63.5
Mean	32.04	31.06
Difference	0.98	
Standard deviation of mean	0.904	

*200 ml. urine from each of 36 human subjects selected at random was hydrolyzed at pH 1 for twenty-four hours at room temperature. The neutral chloroform extract was made up to 200 ml. with chloroform and divided into two equal portions before being concentrated to dryness. The two concentrates were dissolved in 5 ml. methyl alcohol and 5 ml. ethyl alcohol, respectively. One milliliter aliquot of each was taken for the determination. The values are expressed in micrograms desoxycorticosterone equivalent per 40 ml. urine.

and phosphomolybdate reducing methods. The periodic acid oxidation method will liberate formaldehyde from alpha-diols such as Reichstein's Compounds A, E, U, T, and K which are considered to be biologically inactive.

The phenylhydrazine method is simple in operation, but it is suitable only for Kendall's Compounds E and F and is insensitive to a number of other steroids. For example, corticosterone, reported to be a major constituent in blood,[11, 12] produces only $\frac{1}{20}$ as much color with phenylhydrazine as do Compounds E and F.[2]

The colors produced by different cortical hormones with BT, expressed in micromolar quantities, are within a narrow range. It is possible to use the same standard, with some allowances, for the entire series of hormones without introducing any great error. One may apply multiplication factors when the exact value seems desirable. The sensitivity, simplicity, and little difference in color production by all of the cortical hormones tested also make the described tetrazolium techniques useful in the chromatographic separation of reducing steroids.

The purity of the steroids used in this study was not established in our laboratory since they were obtained in small amounts through the courtesy of several laboratories. 17 α-hydroxy-corticosterone (Kendall's Compound F) obtained from three different sources gave widely different values. One sample gave a higher reading, while the other two gave lower readings, per molar equivalent, than 17 α-hydroxy-11-dehydrocorticosterone (Kendall's Compound E). A slight discrepancy due to impurity may be expected in some instances. Nevertheless, it is felt that the color readings are rather close approximations of the true values.

SUMMARY

A simple sensitive procedure for the estimation of reducing steroids by means of blue tetrazolium has been presented. The use of different alkalies and solvents with this reagent is described and applied to the quantitation of small amounts of steroids.

We are indebted to the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.; Ciba Pharmaceutical Products, Inc., Summit, N. J.; The Glidden Company, Chicago, Ill.; G. D. Searle & Co., Skokie, Ill.; and The Upjohn Company, Kalamazoo, Mich., for their generosity in supplying the steroids investigated.

The authors wish to thank Bernice March Katz for her technical assistance.

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A COMPARISON OF THE TETRAZOLIUM AND OTHER METHODS OF STEROID ESTIMATION APPLIED TO URINE

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HINES, ILL., AND CHICAGO, ILL.

SEVERAL investigators have proposed methods of extraction[1, 2, 3, 5, 7, 9] and color estimation[1, 2, 3, 4, 6, 8, 9, 10] of the urinary corticoids in order to avoid the tedious and more elaborate biological assays.[9, 15] Comparable data by the various available methods of corticoid estimation are incomplete, and one is limited in making any comparison because the methods used for extraction vary. The present study was undertaken to provide a comparison of methods using the same extraction procedure in all instances. The methods selected for comparison include that of Heard and associates[4] (phosphomolybdate reduction), Talbot and co-workers[8] (copper reduction), Hollander and collaborators[5] (formaldehyde formation), as well as the reduction of tetrazolium recently proposed by Chen and Tewell[14] and presented in greater detail in the preceding paper. The method of extraction in all instances was essentially that of Heard and colleagues[10] with minor modifications including the use of chloroform rather than a mixture of chloroform and ether.

The method of color development is detailed only as applied to the use of tetrazolium salts. The optimal conditions for corticoid estimation by tetrazolium are presented in the preceding paper. A comparison of the various methods of reducing steroid estimation was made on normal individuals and on patients with normal and abnormal corticoid excretion.

EXPERIMENTAL PROCEDURES AND METHODS

Initially consideration was given to factors concerned in steroid extraction and color development. The effect of pH on the extraction of corticoids from portions of a pooled urine sample was determined. Aliquots were adjusted to various pH values and allowed to stand for twenty-four hours at 25° C. It was found that the corticoids were extractable in greater yield in acid solution (see Table I). Constant values and a high yield were obtained at a pH of 1. At a higher hydrogen ion concentration the urine darkens to a marked degree and additional pigment is carried over into the purified extract. Although the extractable steroids were greater at a pH of 0.5, the results were also more variable. A somewhat greater recovery of corticoids can be obtained by continuous extraction at pH 1.0 with methylene chloride over a period of twenty-four hours, but the results by manual extraction are just as reproducible and the yield is only slightly less (see Table II).

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This research was partially supported by a United States Public Health Service Grant.
Received for publication, June 2, 1953.

TABLE I. EFFECT OF pH CONCENTRATION ON THE EXTRACTION OF URINARY REDUCING STEROIDS

pH OF URINE	NUMBER OF SAMPLES	TETRAZOLIUM BLUE REDUCING STEROIDS EXPRESSED AS GAMMA DESOXYCORTICOSTERONE PER 100 ML.
8.0	6	26.0 ± 3.16
7.0	6	27.0 ± 1.29
6.0	6	28.0 ± 3.34
5.0	6	28.5 ± 0.44
4.0	6	34.0 ± 0.36
3.0	6	36.0 ± 0.41
2.0	10	70.0 ± 0.51
1.0	12	135.0 ± 0.06
0.5	12	200.0 ± 0.70

TABLE II. COMPARISON OF CONTINUOUS AND MANUAL EXTRACTION PROCEDURES

SAMPLE NO.	SEX	MANUAL EXTRACTION MG. TBRS/24 HR.	CONTINUOUS EXTRACTION MG. TBRS/24 HR.	DIFFERENCE MG. TBRS/24 HR.	INCREASED YIELD %
1.	M	1.61	1.78	0.17	10.56
2.	M	0.92	1.01	0.09	9.78
3.	M	1.74	1.92	0.18	10.34
4.	M	1.54	1.69	0.15	9.74
5.	M	1.83	2.01	0.18	9.84
6.	M	1.45	1.58	0.13	8.97
7.	F	1.40	1.53	0.13	9.29
8.	F	1.23	1.35	0.12	9.76
9.	F	1.58	1.73	0.15	9.49
10.	F	1.28	1.40	0.12	9.38
11.	F	0.90	1.00	0.10	10.00
12.	F	1.20	1.31	0.11	9.17

An additional test of the extraction procedure was made by studying the recovery of desoxycorticosterone added to urine and water. In these experiments crystalline desoxycorticosterone dissolved in 1 ml. of ethyl alcohol was added to 100 ml. aliquots of urine. The pH was adjusted to 1.0 and the mixture was allowed to stand for twenty-four hours at 25° C. The results shown in Table III indicate that usually 96 to 101 per cent of the desoxycorticosterone could be recovered.

TABLE III. RECOVERY OF DOC* BY MANUAL EXTRACTION WITH CHLOROFORM

DESOXYCORTICOSTERONE ADDED (GAMMA)	FLUID VOLUME	NUMBER OF SAMPLES	RECOVERY RANGE (PER CENT)
25	100 ml. water	4	96-101
50	100 ml. water	4	97- 99
100	100 ml. water	4	97-101
25	100 ml. urine	4	96- 99
50	100 ml. urine	4	98-101
100	100 ml. urine	4	96-100

*Desoxycorticosterone.

Studies on the rate and extent of color development of various steroids with BT are included in the preceding paper. According to these results the BT color developed by the adrenocortical steroids in methanol:NaOH stabilizes after eighteen minutes and remains constant for at least two hours.

The reagents employed and routine procedure for the extraction of urine and development of color follow:

Reagents.—Chloroform—C.P. reagent grade, Mallinckrodt

0.1N Sodium hydroxide—4 Gm. of C.P. reagent grade, NaOH pellets are dissolved in 1,000 ml. of distilled water. A large volume of this solution is prepared. Aliquots are chilled in the refrigerator before use.

Hydrochloric acid—Concentrated, reagent grade, C.P.

Sodium sulfate—Anhydrous, granular, C.P. reagent grade

Methyl alcohol—Absolute, reagent grade, C.P., Mallinckrodt, distilled from dinitrophenylhydrazine. Fraction distilled between 63.5° C. and 65° C. is collected.

Blue tetrazolium (BT)—Ditetrazolium chloride. Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, and Manomer-Polymer, Inc., Leominster, Mass. It is important that this dye be purified before use. The pure compound is pale yellow in color and melts at 245° C. with decomposition. If the compound does not meet this standard of purity it is necessary that it be recrystallized in the following manner: the impure tetrazolium salt is dissolved in methanol with the aid of heat and ethyl acetate in acetone is added until crystals form. The yellow crystals are collected and dried. This process is repeated until a crystalline product is obtained that melts at 242 to 245° C. dec.[12]

Blue tetrazolium (BT) 1 per cent methanolic solution—The amount of dye desired is weighed and transferred to a volumetric flask with the aid of small amounts of redistilled absolute methanol. The solution is then diluted to volume and thoroughly mixed. This solution is prepared the day it is to be used and is stored in an amber glass-stoppered bottle.

Pyridine—Dried over barium oxide and distilled. Only the middle fraction of the distillate is saved.

Acid-pyridine—20 ml. of concentrated hydrochloric acid is added with care to 100 ml. of freshly distilled pyridine. Cool before use. (Prepare acid-pyridine solution under a hood.)

Methanol-acid-pyridine solution—3 parts of methanol are mixed with 1 part of acid-pyridine the day this reagent is to be used.

1.0N Sodium hydroxide in 80 per cent methanol—40 Gm. of reagent grade sodium hydroxide pellets are dissolved in 200 ml. of double distilled water. Cool and dilute to 1,000 ml. with redistilled absolute methanol. Mix thoroughly. This solution is titrated against standard acid and adjusted to exactly 1.0N.

Standard stock solution—25.0 mg. of crystalline desoxycorticosterone in 25.0 ml. of redistilled absolute methanol. Store in tightly stoppered dark bottle in the refrigerator.

Working standards—Stock standard diluted in 1:10. Concentrations of 10, 20, 40, 60 and 100 gamma per ml. in redistilled absolute methanol are used to establish a standard reference curve.

Extraction and Purification of Total Urinary Corticosteroid Extract.—The general plan of extraction is indicated in the accompanying flow sheet (Fig. 1).

Colorimetric Estimation of Total Urinary Corticoids by the Reduction of the Blue Tetrazolium (BT) Dye.—All solutions are brought to a temperature of 25° C. before use. The reaction is carefully timed from the addition of the tetrazolium blue solution to each tube.

1. *Standard Solution:* A standard reference curve is prepared by using known solutions of desoxycorticosterone in absolute methanol containing 10 to 100 gamma per milliliter. Prepare colorimeter tubes in duplicate, containing 1 ml. of desoxycorticosterone standard in redistilled absolute methanol. To each add 1 ml. 1N NaOH in 80 per cent methanol and 1 ml. 1 per cent blue tetrazolium (BT) in absolute methanol.
2. *Reagent Blank:* Prepare colorimeter tubes in duplicate, each to contain 1 ml. redistilled absolute methanol, 1 ml. 1N NaOH in 80 per cent methanol, and 1 ml. 1 per cent blue tetrazolium (BT) in absolute methanol.

3. *Urine Extract:* In another series of tubes, prepared in duplicate, the purified steroid extracts in methanol solution are substituted for the standard solutions. Each tube contains 1 ml. steroid extract in methanol, 1 ml. 1N NaOH in 80 per cent methanol and 1 ml. 1 per cent blue tetrazolium (BT) in absolute methanol.
4. *Color Correction for Urine Extract:* These tubes may be prepared for each urine extract, but are actually only necessary for colored steroid extracts. To each tube add 1 ml. redistilled absolute methanol, 1 ml. 1N NaOH in 80 per cent methanol and 1 ml. 1 per cent blue tetrazolium (BT) in absolute methanol. The color correction tubes need not be incubated twenty minutes. Acid-pyridine 1.0 ml. is added to each tube followed by the addition of 1.0 ml. steroid extract and 2 ml. absolute methanol. The tubes are well mixed and tightly stoppered. The results expressed as mg. steroid are subtracted from the total value for the urine extract.
5. *Color Development:* The details of color development are presented in the text of the preceding paper.

Results are expressed as milligram equivalents of desoxycorticosterone excreted per twenty-four hours. A standard calibration curve is used. This curve is checked with each group of unknowns by reacting standard solutions containing 20, 40, and 60 gamma per milliliter of absolute methanol. The conversion factor for expressing results as compound F is 1.3.

DISCUSSION

The physiologically active steroids all have the alpha-beta unsaturated 3-ketone in ring A of the steroid nucleus and each has an alpha-ketol side chain. These compounds differ only in the degree of oxygenation at C-11 and in the fact that compounds S, F, and E have an alpha-hydroxyl group at C-17. The C-21 side chain is extremely sensitive to acids, alkalies and oxidizing agents. The analytical techniques employed utilize both the solubility and chemical characteristics of these steroids. The specificity of the procedure depends largely upon the complete removal of interfering reducing substances that may react with the reagent.

The use of chloroform as a solvent for steroid extraction has been discouraged by some authors[10] because of its tendency to form stable emulsions with urine. However, the use of cold alkali and water for removal of interfering substances from the chloroform extract enables one to use this solvent without difficulty. The neutral lipid extract must be reduced to dryness at a low temperature (less than 50° C.) to avoid decomposition of the reducing steroids. Further fractionation of this extract prior to color development has been reported by Romanoff and co-workers[16] but is not considered necessary for routine purposes. More specific information regarding individual compounds may be obtained by partition between benzene and water or by means of Girard's reagent T.[8]

The actual corticoid content of urine must be much greater than the amount obtained after acid hydrolysis. Investigators[17, 18, 19] have shown that the chemical hydrolysis of conjugated steroids is destructive of the steroid. The greatly increased yield of urinary corticoids following hydrolysis with beta-glucuronidase[17, 18, 19] indicates the inadequacy of the purely chemical procedure. Elevating the temperature of the acidified urine destroys the physiologically active adrenal steroids.[17] The greater yield of corticoids at lower pH values is in

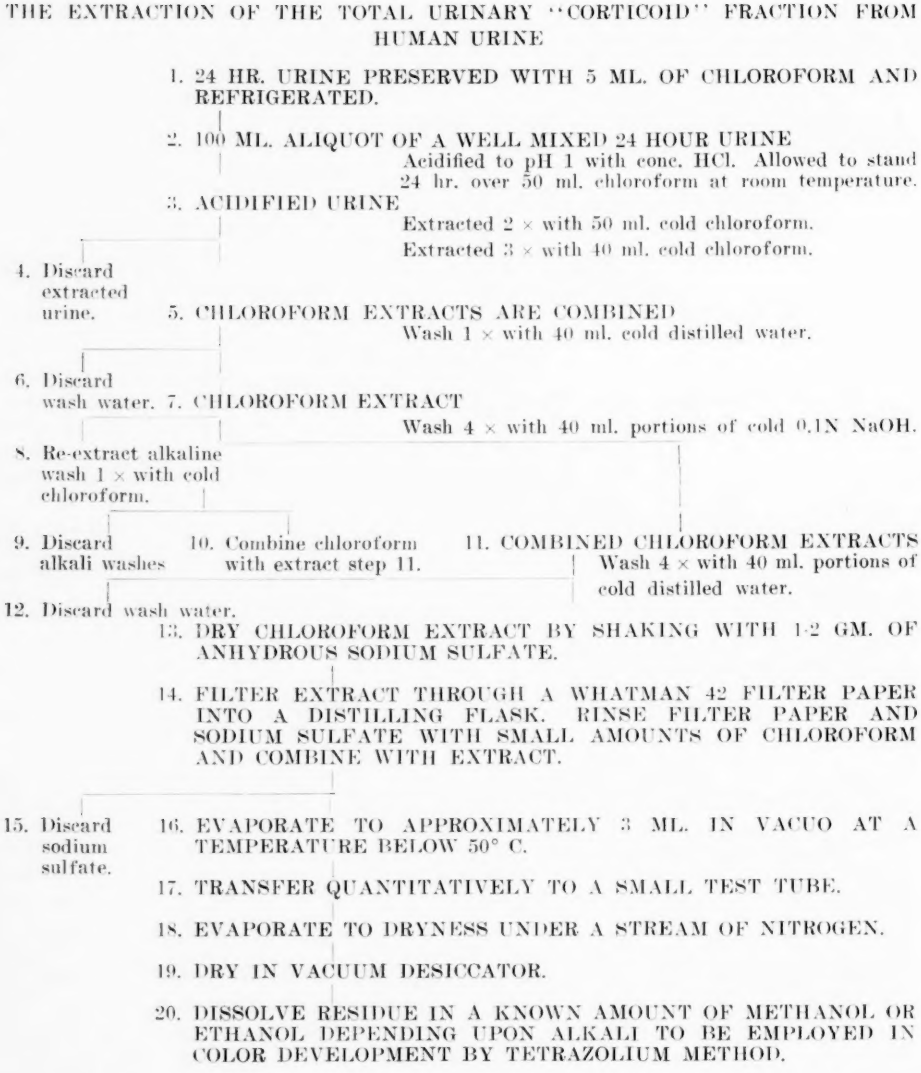
THE EXTRACTION OF THE TOTAL URINARY "CORTICOID" FRACTION FROM HUMAN URINE
1. 24 HR. URINE PRESERVED WITH 5 ML. OF CHLOROFORM AND REFRIGERATED.
2. 100 ML. ALIQUOT OF A WELL MIXED 24 HOUR URINE
Acidified to pH 1 with conc. HCl. Allowed to stand 24 hr. over 50 ml. chloroform at room temperature.
3. ACIDIFIED URINE
Extracted 2 × with 50 ml. cold chloroform.
Extracted 3 × with 40 ml. cold chloroform.
4. Discard extracted urine.
5. CHLOROFORM EXTRACTS ARE COMBINED
Wash 1 × with 40 ml. cold distilled water.
6. Discard wash water.
7. CHLOROFORM EXTRACT
Wash 4 × with 40 ml. portions of cold 0.1N NaOH.
8. Re-extract alkaline wash 1 × with cold chloroform.
9. Discard alkali washes
10. Combine chloroform with extract step 11.
11. COMBINED CHLOROFORM EXTRACTS
Wash 4 × with 40 ml. portions of cold distilled water.
12. Discard wash water.
13. DRY CHLOROFORM EXTRACT BY SHAKING WITH 1-2 GM. OF ANHYDROUS SODIUM SULFATE.
14. FILTER EXTRACT THROUGH A WHATMAN 42 FILTER PAPER INTO A DISTILLING FLASK. RINSE FILTER PAPER AND SODIUM SULFATE WITH SMALL AMOUNTS OF CHLOROFORM AND COMBINE WITH EXTRACT.
15. Discard sodium sulfate.
16. EVAPORATE TO APPROXIMATELY 3 ML. IN VACUO AT A TEMPERATURE BELOW 50° C.
17. TRANSFER QUANTITATIVELY TO A SMALL TEST TUBE.
18. EVAPORATE TO DRYNESS UNDER A STREAM OF NITROGEN.
19. DRY IN VACUUM DESICCATOR.
20. DISSOLVE RESIDUE IN A KNOWN AMOUNT OF METHANOL OR ETHANOL DEPENDING UPON ALKALI TO BE EMPLOYED IN COLOR DEVELOPMENT BY TETRAZOLIUM METHOD.

Fig. 1.—Flow sheet indicating the sequence of events in the extraction and purification of urinary neutral reducing steroids.

accord with the data of others[10] who have shown that mild hydrolysis increases the yield of reducing substances. The time and temperature as well as the pH for urine hydrolysis were all carefully controlled in this study.

The formaldehydogenic procedure may be somewhat more specific than the tetrazolium for the side chain characteristics of steroids. This fact explains the slightly lower values obtained on normal subjects and in various pathological states with the formaldehydogenic procedure. The phosphomolybdate reagent

is evidently less specific than the other reducing substances employed. Romanoff and associates[16] have pointed out that total urinary corticosteroid extracts contain about 20 per cent of compounds that are classified as "nonalcoholic reducing lipids" which are probably not adrenal in origin. The copper reduction method gives results that are practically identical with the tetrazolium method but the procedure is more tedious and time consuming. The variations in corticoid excretion obtained in normal (Table IV) and disease states by the different methods parallel one another closely (Table V).

TABLE IV. RANGE OF CORTICOID EXCRETION BY NORMAL ADULT HUMAN SUBJECTS

SUBJECT	AGE	SEX	TBRS	"CORTICOIDS"*		FS
				PRS	CRS	
1.	33	M	1.75	2.00	1.80	1.59
2.	30	M	2.07	2.12	2.10	1.88
3.	36	M	1.61	1.80	1.66	1.46
4.	29	M	1.83	2.00	1.90	1.65
5.	34	M	1.74	1.90	1.81	1.57
6.	30	M	0.92	1.10	1.05	0.81
7.	25	M	1.78	1.90	1.83	1.60
8.	32	M	1.00	1.16	1.10	0.91
9.	60	M	1.54	1.68	1.60	---
10.	29	M	1.25	1.35	1.30	---
11.	31	M	2.45	2.80	2.45	---
12.	30	M	1.70	1.92	1.73	1.52
13.	26	M	1.25	1.40	1.29	1.12
14.	32	M	1.20	1.33	1.26	1.09
15.	33	M	1.12	1.20	1.17	1.01
16.	31	M	1.35	1.46	1.40	1.15
17.	36	M	1.70	1.81	1.73	1.60
18.	40	M	1.45	1.59	1.51	1.30
19.	55	F	1.97	2.40	2.24	1.75
20.	45	F	1.40	1.50	1.46	1.25
21.	34	F	1.63	1.80	1.70	1.45
22.	29	F	1.58	1.71	1.66	1.40
23.	24	F	1.28	1.40	1.32	---
24.	30	F	0.90	1.15	1.03	---
25.	28	F	1.20	1.35	1.28	1.07
26.	30	F	1.50	1.71	1.60	---
27.	26	F	1.42	1.60	1.52	1.25
28.	54	F	1.42	1.58	1.50	---
29.	24	F	1.25	1.45	1.33	---
30.	35	F	1.70	1.83	1.75	---
31.	26	F	1.10	1.22	1.15	0.98
32.	22	F	1.04	1.26	1.12	0.90
33.	31	F	0.98	1.16	1.04	0.89
34.	28	F	1.12	1.30	1.19	1.00
Average adult males			1.54 ± 0.38	1.70 ± 0.41	1.59 ± 0.36	1.35 ± 0.31
Average adult females			1.34 ± 0.28	1.53 ± 0.47	1.43 ± 0.31	1.20 ± 0.20

*Milligram excreted per twenty-four hours expressed as the reducing equivalent of desoxycorticosterone. TBRS, Tetrazolium blue reducing steroid. PRS, Phosphomolybdate reducing steroid. CRS, Copper reducing steroid. FS, Formaldehydogenic steroid.

It is apparent from the results presented that similar results may be obtained by all four methods. However, the simplicity and stability of the reagents required for the tetrazolium procedure as well as the great stability of the color developed by its reduction make this method preferable in the opinion of the authors.

TABLE V. AVERAGE "CORTICOID" EXCRETION IN HEALTHY SUBJECTS AND IN VARIOUS DISEASE STATES

CONDITION:	NUMBER OF SUBJECTS	SEX	AGE RANGE (YR.)	NUMBER OF DET'NS	AVERAGE EXCRETION OF "CORTICOIDS"* TBRS	PRS	CRS	FS
Normal adult males	18	M	24-61	18	1.54	1.70	1.59	1.35
Normal adult females	16		22-55	16	1.34	1.53	1.43	1.21
Hospitalized adult males	150		25-60	150	1.44	1.68	1.56	1.31
Addison's disease	1	M	29	8	0.43	0.58	0.50	0.39
	1	M	32	8	0.29	0.49	0.41	0.26
	1	M	38	3	0.40	0.56	0.48	0.36
	1	M	39	3	0.48	0.61	0.56	0.44
Adrenal adenocarcinoma:	1	M	34	10	3.46	5.09	3.81	3.15
	1	M	32	11	4.24	5.31	4.50	3.85
Hypopituitarism:	1	M	40	6	0.50	0.70	0.61	0.46
	1	M	36	4	0.78	0.92	0.83	0.69
	1	M	62	9	0.32	0.39	0.34	0.28
	1	M	60	8	0.33	0.40	0.36	0.29
Cushing's syndrome:	1	M	26	4	3.73	5.10	4.05	3.35
Chronic inanition:	1	F	31	3	0.34	0.48	0.40	0.30
	1	M	38	3	0.81	1.03	1.00	0.72
	1	M	58	3	0.85	1.09	1.01	0.76
Pregnancy:								
Normal excretion	1	F	28	2	1.20	1.35	1.28	1.07
2nd month of gestation				2	1.66	1.85	1.78	1.48
4th month of gestation				2	1.80	2.04	1.92	1.60
6th month of gestation				2	2.05	2.45	2.15	1.79
8th month of gestation				2	2.95	3.20	3.05	2.60
2 months postpregnancy				2	1.50	1.76	1.63	1.33

*Milligram "Corticoids" excreted per twenty-four hours expressed as the reducing equivalent of desoxycorticosterone. TBRS, Tetrazolium blue reducing steroid. PRS, Phosphomolybdate reducing steroid. CRS, Copper reducing steroid. FS, Formaldehydogenic reducing steroid.

SUMMARY

The extraction and purification of urinary neutral reducing steroids has been described. Reducing steroid estimation by means of blue tetrazolium has been detailed and compared to other procedures. It gave similar results on normal and pathological specimens and has numerous advantages from the standpoint of analytical technique.

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STABLE PRECISION IN A READILY ASSEMBLED, CONTINUOUSLY RECORDING PHOTOELECTRIC BUBBLE FLOW METER

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SOSKIN and associates[1] introduced the bubble flow meter for blood flow measurements. General considerations of principles, advantages, and limitations of such instruments have been given by Bruner.[2] Selkurt[3, 4] introduced photoelectric recording of the bubble passage time, thus removing subjective inaccuracy. This, in turn, allows shortening of the tube length and bubble passage time, hence reduction of pressure loss and alleviation of the temperature control problem. Sokalchuk and co-workers[5] employed the photoelectric signal electromechanically to reverse the direction of flow in the meter upon arrival of the bubble at either signal station, thus confining the bubble to movements back and forth between stations, and providing for continuing recording of flow rate after injection of a single bubble. A similar principle had been applied by others to the Ludwig type Stromuhr.

The Sokalchuk instrument involves dual custom-built photoelectronic drivers to operate the reversing solenoids. It may be useful, therefore, to describe an implementation of automatic photoelectric bubble reversal that uses a single, inexpensive, commercial photoelectronic unit and that is assembled largely from stock components. The apparatus has been in relatively carefree use for about two years.

MATERIALS AND PROCEDURES

Referring to Fig. 1, drawn to scale, the mechanical assembly is in two units: *A* consists of the bubble-carrying tube and the photoelectric station, and *B* is an electromechanical valve for reversing the flow in *A*.

Looking first at unit *A*, the central feature is an aluminum block, *a*, through which two light ports, *b*, and two channels for a polyethylene tube, *c*, are drilled. Three rods are fitted tightly into holes in this block: *d* is a support rod for the entire unit, and the two *e* rods serve both to support a plastic shelf, *f*, bolted to them, and to aid in shaping the loop of the tube at points *g*. The shaping of the loop is completed by means of the stick, *h*.

Bolted to the under surface of the aluminum block is the photocell box, *j*. It was convenient to cut down the box of a Fisher* Phototube Unit so that the sensitive element of the tube (RCA-921) was centered immediately below the two light ports, *b*. Light leaks in the box were eliminated with masking tape.

At *k*, two opposed signal magnets† working on a common armature operate a shutter over the light ports, and are mounted on a separate rod.

The light source is not shown. A Spencer Universal microscope lamp, model No. 353,‡ is slipped from its base and mounted with an ordinary clamp to the same stand that supports unit *A* and the shutter assembly. With the lens about 15 cm. over the light ports, the beam conveniently adjusts to a sharp rectangular area just covering the two ports.

From the Research Laboratories, Parke, Davis and Company.
Received for publication, June 26, 1953.

*Fisher Scientific Co., Cat. No. 13-991-5 (1952).

†*E.g.*, Arthur H. Thomas Co., Cat. No. 8063 (1950).

‡*E.g.*, Harshaw Scientific, Cat. No. H-50,420 (1950).

The other major mechanical unit, *B*, the electromagnetic reversing valve, is mainly a product of the laboratory scrap bin; it may well vary considerably according to available materials. The opposed solenoids illustrated, *p*, happen to be Allen-Bradley,* series 5PPUA, RC3501, 110 V., 60 cycle. They provide an excess of power, and greater finesse could perhaps be achieved by use of smaller units, particularly if DC-powered. To prevent AC-chatter, their mounting must be adjusted rather carefully with respect to full, tube-occluding positions of the clamp head, *q*.

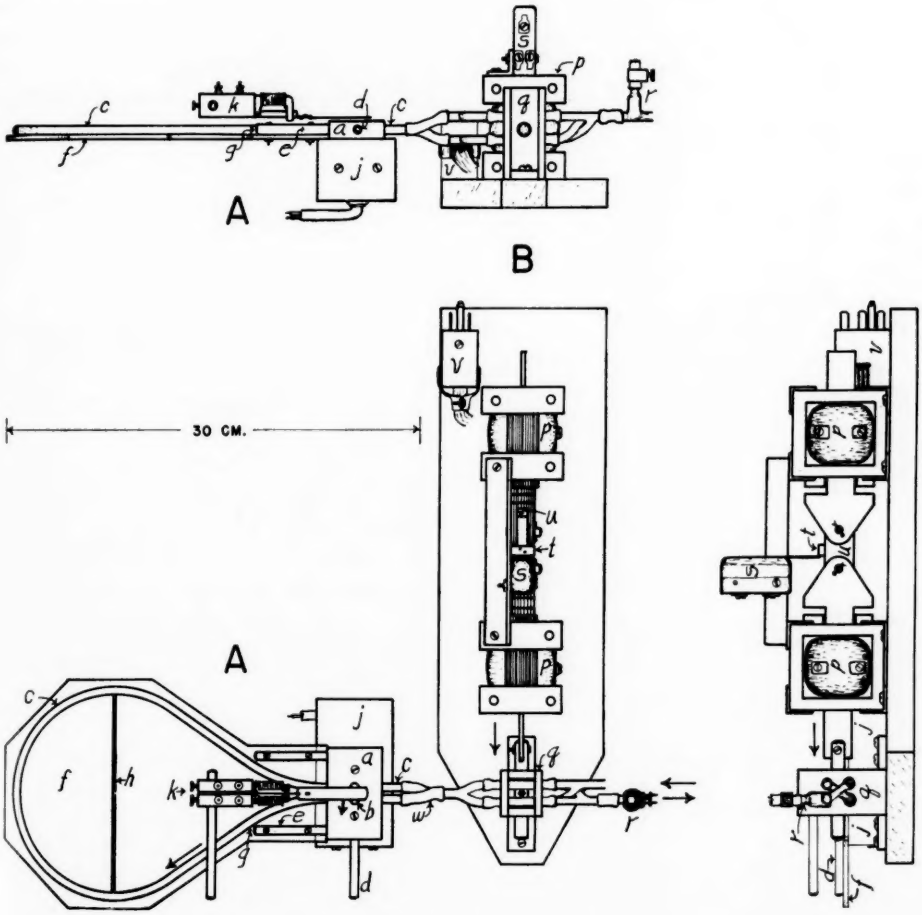

Fig. 1.—Top (lower left), side (upper) and end (right) views of *A*, the bubble-carrying loop and photocell station, with phasing shutter, and *B*, the reversing valve. Small case letters refer to parts described in the text. The lamp and certain stock electrical equipment are not shown.

The clamp head, *q*, is constructed from available pieces of brass that suggested the design shown. Four pieces of pure gum tubing threaded through supporting holes in the brass head, together with four glass Y's, complete the reversing valve. The entire linkage is mounted on a sturdy piece of hardwood, in turn bolted to a heavy metal shelf independent of other apparatus to avoid transmission of the solenoid jar. Connections are made between the valve

*Allen-Bradley Co., Milwaukee, Wis.

and unit *A* and the animal, through shortest reasonable lengths of pure gum tubing. A suitable air trap, *r*, as well as a manometer connection (not shown) are included in the return line to the animal, and a calibration by-pass is connected across inlet and outlet.

The movement of a block, *t*, fastened to the armature connecting link, *u*, operates a single-pole double-throw (BZ-2RW-A2) Micro Switch* at *s*. This controls the shutter, *k*, and a recording signal magnet not shown in Fig. 1. All electrical connections to assembly *B* are made through the connector *c*.

Supplemental electrical components used are a Fisher-Serfass Electronic Relay, Improved,† companion-piece of the Phototube Unit; a Struthers Dunn‡ ratchet relay, type (A) 11BXX, 115 volt 60-cycle coil, 115 volt AC 6 ampere contacts; and a 3 volt DC source for operating the signal magnets. These are all placed in the wiring diagram, shown in Fig. 2.

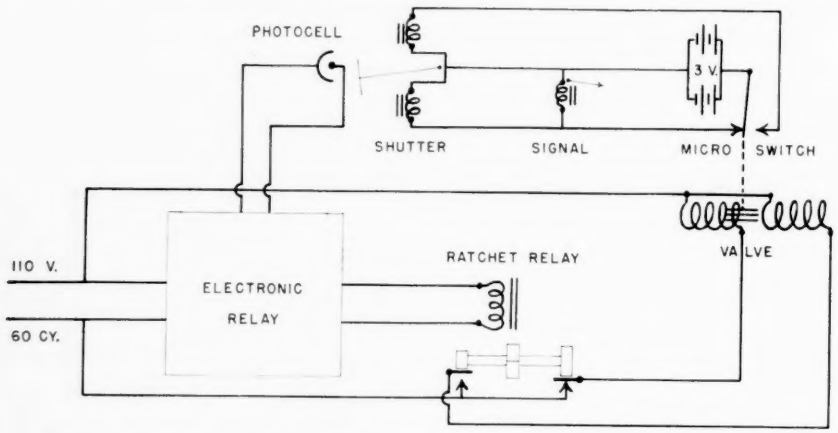

Fig. 2.—Wiring diagram. Specifications of components are given in the text.

All tubing is wrapped with cotton to reduce heat loss. After an experiment connections are broken at convenient points and all tubing brushed and rinsed thoroughly. Reassembled, the tubing is filled with heparinized physiological saline solution before connection to an animal for use again.

In the first trial, connections to the shutter magnets are reversed, if necessary, until one of the two positions of the ratchet relay provides the valve and shutter positions and flow direction indicated by arrows in Fig. 1. With blood flowing through the apparatus, an air bubble injected into the tube *w* through an ordinary syringe and fine needle travels uneventfully past the photocell station because the shutter is over the respective light port. On arrival at the opposite light port, light is transmitted to the photocell, the electronic relay is activated, the ratchet relay reverses contacts, and thus the activity of the opposed solenoids is reversed. Simultaneously, therefore, the bubble starts moving in the opposite direction, and the Micro Switch reverses the shutter position to cover the port just traversed by light and signals the event on the kymograph. The other half of the cycle is then completed analogously. It is seen that the shutter serves to insure against any accidental repetitive photoelectric activity while the bubble is in station, which, if repeated an even number of times, would send the bubble on out the exit to the trap. For this reason we call the shutter the "phaser."

*Micro Switch, Freeport, Ill.
†Fisher Scientific Co., Cat. No. 13-991 (1952).
‡Struthers Dunn, Inc., Philadelphia, Pa.

RESULTS AND CRITIQUE

Inasmuch as the volume of connections between the photocell station and the reversing valve is less than that of the bubble loop, blood about the bubble is constantly being refreshed, providing continuing gaseous exchange. Hence, as pointed out by Sokalchuk and associates,[5] a bubble which is thus kept oscillating may gradually diminish in size. This is particularly the case when the animal has been subjected to nitrogen "wash-out," as during artificial ventilation with oxygen. In six such consecutive experiments the bubble shortened at an average rate of 10 mm. in seventeen to forty-five minutes (average, thirty-five minutes). During natural respiration of room air, on the other hand, the bubble may change much more slowly, or, indeed, imperceptibly over long periods. We have arbitrarily allowed an old bubble to escape when it is reduced to about 10 mm. in length. This is done by manually shading the light port as the bubble approaches it. A new bubble 20 to 25 mm. long is then injected on the opposite (shuttered) side.

It will be apparent that in the case of such an oscillating bubble a given actual flow rate will be represented by an oscillation (reversal) frequency depending in part on bubble length. It is therefore necessary for utmost accuracy to estimate the actual bubble length and adjust the recorded oscillation frequencies to that of some standard bubble length. Thus, if the loop of tubing between photoelectric stations is 60 cm. long and if 20 mm. is taken as the arbitrary standard bubble length, the adjustment factor is

$$F = \frac{(600 \text{ mm.}) - (\text{actual bubble length in mm.})}{600 \text{ mm.} - 20 \text{ mm.}}$$

A table of such factors for estimated actual bubble lengths of 5 mm. to 27 mm. in 1 mm. steps, together with a slide rule or automatic desk computor, renders the adjustment sufficiently convenient. However, a 10 mm. deviation in bubble length, above or below standard, will be seen to call for correction by a factor of only 1.017 or 0.983, as the case may be. Hence, within a reasonable range of bubble lengths, and with a loop this long, the adjustment may be ignored without serious consequence. Error in estimation of total *effective* loop length *(infra)* is readily seen to be of vanishing importance as it concerns computation of the adjustment factor.

Effective loop length is another concept peculiar to oscillation of the bubble. In the case of the conventional unidirectional bubble flow meter, errors in timing of arrival of the bubble front at the two marks, whether visual or photoelectric, are likely to cancel out on the average. When the bubble is made to oscillate between the stations, however, these potential errors at the opposite ends of its run are additive and depend on total photoelectromechanical lag in reversing the loop flow. The shorter the time of oscillation, as the flow rate increases, the greater the relative importance of this potential error. Thus, empirical calibration becomes more desirable, and it is fortunate that convenience of such calibration is another peculiarity of the continuously oscillating and recording bubble.

In Fig. 3 are plotted all the in vivo calibration data for the instrument as now used. The polyethylene loop measures about 60 mm. between stations and has an inside diameter of 4.0 mm.* The three sets of data shown were obtained with the flow meter connected into the course of the common carotid artery of respective anesthetized, heparinized dogs. Each point entails (a)

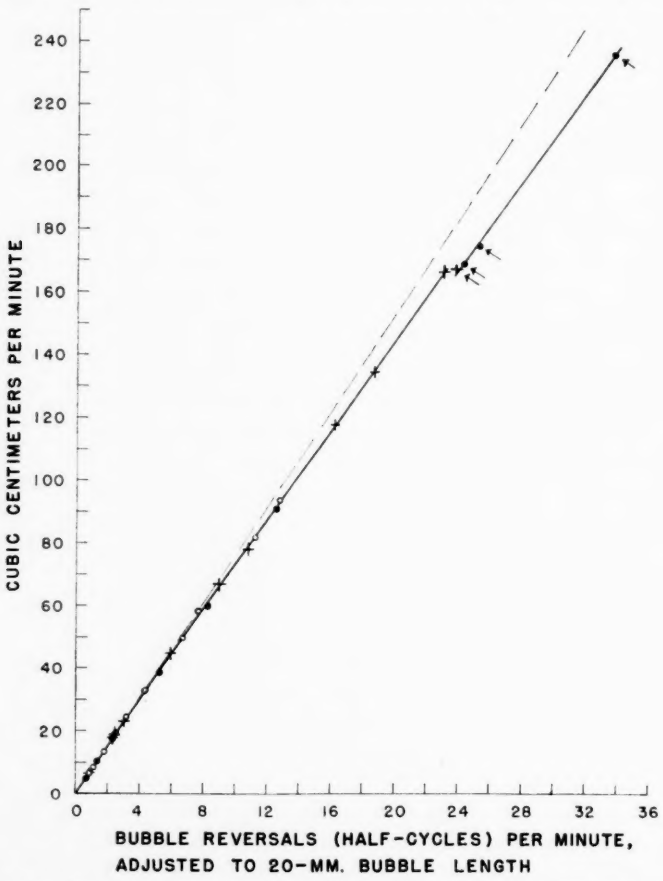

Fig. 3.—Calibration curve, showing all observations in all three such experiments, and covering an eight-week interval during which the lamp bulb was renewed. Points indicated by arrows show the influence of a large, deliberate change in light intensity. The dashed straight line assumes uniform flow per reversal equal to the average of the (adjusted) values of the five lowest flow rates (7.65 c.c. per reversal).

opening the calibration by-pass, (b) closing the return line between the by-pass and the meter, (c) opening to the desired flow a side tube (air trap escape) between this point of closure and the meter, (d) collecting this outflow into a graduated cylinder for an integral number of bubble reversals, and, finally, (e) reinfusing the collected blood into the animal. In the neighborhood of 50 c.c. to 100 c.c. were collected during 7 to 13 (usually 12) bubble reversals. Time in seconds and bubble reversals were recorded kymographically.

*Clay-Adams PE-380.

The shift in the calibration line representing the upper four points is due to an increase in the light intensity to maximum and removal of a heat filter. This was necessary to prevent escape of the bubble past the photoelectric station at these high flows, and indicates that there was, indeed, photoelectric lag, at least with the lower light intensity. Up to a flow rate of 60 to 70 c.c. per minute, however, any resulting increase in *effective* loop length was insufficient to overcome the normally expected gradual decrease in slope of the calibration curve due to the bubble running ahead of the flow (Bruner[2]). Beyond 60 to 70 c.c. per minute the rates of change in the two opposing influences about balanced one another to produce a substantially straight line. The large, deliberate change in photoelectric conditions shifted the flow per bubble reversal by less than 4 per cent, at a flow rate of 160 to 166 c.c. (point of break). If utmost precision in absolute flow measure is needed, the instrument is easily calibrated occasionally as a check on photoelectric stability. It may be noted in this connection that (a) the first and last experiments plotted were separated by eight weeks, (b) that a new lamp bulb was installed between the first and second, and yet, (c) that the scatter of individual points from the line easily could be accounted for by errors in collecting blood in the graduate and reading its volume.

Blood cell damage due to tube occlusion in the reversing valve has not been studied.

There remains a final potential disadvantage peculiar to oscillation of the bubble. A mass of blood is repeatedly deaccelerated to zero and reaccelerated in the opposite direction or in other tubing (in the valve). In the case of the particular dimensions being considered, the involved blood mass amounts to about 15 to 20 Gm., and the velocity to as much as 16 cm. per second at a flow rate of 100 c.c. per minute. The energy change is a very transient phenomenon whose time course will vary with elastic properties of the system. We have not attempted to record it. At flow rates up to at least 100 c.c. per minute, it can just be detected with certainty on a damped mercury manometer connected to the outflow or inflow line. At such flow rates its contribution to the average pressure drop through the instrument is certainly negligible. However, when an undamped mercury manometer was connected differentially across the input and output lines at the level of the calibration by-pass through wide-bore metal tubing, very transient downward swings of 1 to 5 mm. Hg were seen on valve reversal, at flows up to 100 c.c. per minute. The exact movement depended on the flow rate, the amount of air in the small air trap, and the degree of coincidence with the diastolic phase of the pulse cycle. Inasmuch as the bubble flow meter is not suitable for study of transient flows at best, this transient could be damped almost at will as by increasing the amount of air in an enlarged trap.

Two methods were used to measure the over-all average pressure drop through the flow meter, from and to the level of the calibration by-pass. First, it was read on the above-mentioned differentially connected mercury manometer after damping, with the flow meter connected into the common carotid artery of the dog and with the flow artificially adjusted to various levels by means of

a screw clamp on the outflow line. Secondly, separate damped recording manometers were connected to the respective sides and the pressure drop measured on the record at various flow rates, as the change in the manometers' differential positions relative to their differential at zero flow. The two approaches agreed to a roughly linear relationship between pressure drop and flow in the range from about 1.8 mm. Hg at 10 c.c. per minute to about 10 mm. Hg at 100 c.c. per minute. Such values are not high for a bubble flow meter (cf. Bruner[2], Selkurt[3, 4]). Viscosity determinations were not made, but pressure drop values soon after rapid infusion of 110 c.c. of normal saline indicated no important deviations from the above relationship.

SUMMARY

Precision and stability of calibration and convenience in assembly and use have been described for a recording bubble flow meter in which the bubble is caused to oscillate continuously between two photoelectric stations employing a common photocell. The over-all pressure drop is satisfactory for a bubble flow meter, and the transient energy changes associated with flow reversals are small and can be damped almost at will. With optimal light and optical adjustment flows in excess of 200 c.c. per minute were recorded.

Acknowledgment.—The senior author is grateful to Dr. Sokalchuk for an opportunity to see his instrument in operation.

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IMPROVED INJECTION PIPETTE FOR DETERMINATION OF CARDIAC OUTPUT BY THE DYE METHOD

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ONE of the important steps in determining cardiac output by the dye dilution method is a quick and smooth intravenous injection of a precisely known amount of dye (T-1824) to produce a bolus which ascends in the veins toward the heart and lungs.[1, 2] Various ways of injecting the dye into an antecubital vein or into a catheter in the pulmonary artery have been described. These methods include the use of two syringes with three-way stopcocks, weighing of the dye syringe before and after injection, and calibration of the injection needle to estimate the residual dye.[3-9] The reported techniques of injection incur an error of 2 per cent or more. The weighing and repeated calibration of syringes are time consuming and cumbersome. The necessary interruption between manipulations for injecting and flushing may give rise to inaccurate results. Our experience with these difficulties[2] led to the design of the present special pipette for the purpose of making a rapid injection of a precise amount of dye.

MATERIAL AND METHODS

The pipette consists of a special stopcock with unequal arms and with brass ends fitted and cemented on (Fig. 1). Heavy-walled Pyrex capillary tubing of 3 mm. bore is used and the stopcock plug has also a 3 mm. bore. A uniform cylindrical shape was chosen for the lumen in order to reduce turbulence and thus facilitate flushing and delivery of the dye. Several sizes of pipettes were made so that amounts of dye could be injected according to the body weight of the patient. The volumes are approximately 1.1, 1.5 and 2.2 ml., and the total lengths are 21, 26, and 38 cm., respectively. The pipette attached to a syringe is shown in Fig. 2.

In preparation for the injection the pipette is filled to its calibrated volume with dye. This is done by dipping the tip (*1*, Fig. 1) into the dye, aspirating with a syringe until the dye is above the stopcock, closing the stopcock, flushing out the excess dye from the short arm with sterile normal saline, and wiping any excess dye from the tip. The flushing syringe with 10 ml. of sterile normal saline is fitted into the short arm of the pipette. The tip (*1*, Fig. 1) of the pipette is fitted into a No. 15 intravenous needle which has been placed previously in an antecubital vein of the patient. The injection is made by opening the stopcock and emptying the contents of the flushing syringe through the pipette in a smooth stroke which requires three to four seconds. The stopcock is then closed and the pipette removed. The small amount of dye left within the pipette is washed into a beaker by flushing with

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Aided by a grant from the National Institutes of Health, Public Health Service, No. G-3485(C).

Received for publication, June 29, 1953.

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TABLE I. AVERAGE PERCENTAGE OF RESIDUAL DYE IN THE PIPETTE FROM TWENTY-THREE EXPERIMENTS

EXPERIMENT NO.	TOTAL AMOUNT OF DYE FOR INJECTION (MG.)	DYE LEFT IN THE PIPETTE (MG.)	RESIDUAL DYE (PER CENT)
1	5.70	0.002	0.04
2	5.70	0.017	0.31
3	5.70	0.017	0.31
4	5.70	0.043	0.77
5	5.70	0.058	1.02
6	5.70	0.006	0.11
7	5.70	0.020	0.36
8	5.70	0.019	0.34
9	7.87	0.100	1.27
10	7.87	0.076	0.97
11	7.87	0.060	0.76
12	11.25	0.108	0.96
13	11.25	0.108	0.89
14	11.25	0.100	0.89
15	11.25	0.108	0.96
16	10.90	0.102	0.90
17	10.90	0.071	0.65
18	10.90	0.085	0.78
19	10.90	0.081	0.74
20	8.55	0.074	0.86
21	8.55	0.046	0.54
22	8.55	0.054	0.63
23	8.55	0.046	0.54
		Mean	0.68% ± 0.07%
		S.D.	±0.32%

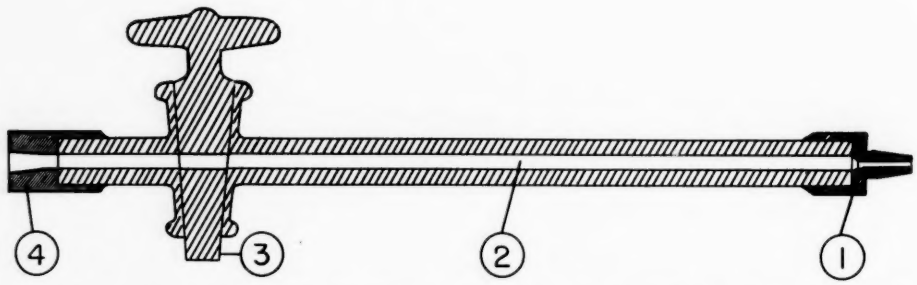

Fig. 1.—Precision dye-injection pipette. Section. *1*, Brass end with male taper to fit hypodermic needle. *2*, Volume—3 mm. bore calibrated through plug of the stopcock to the tip of part 1. *3*, Stopcock. *4*, Brass end female taper to fit syringe. Both ends cemented to the glass with litharge.

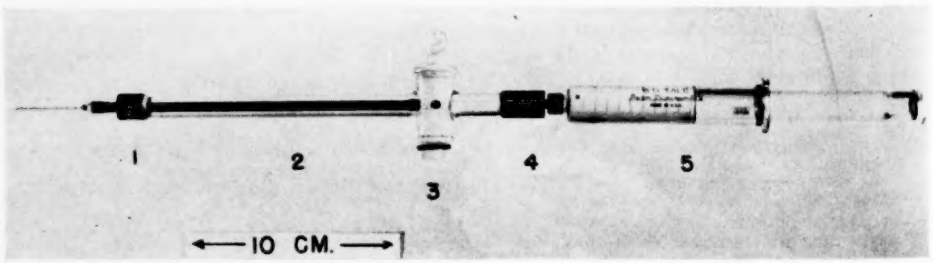

Fig. 2.—Dye pipette and flushing syringe ready for injection. *1*, *2*, *3*, *4*, parts of the precision pipette (see legend Fig. 1). *5*, 10 ml. flushing syringe.

another 10 ml. of saline. The amount of residual dye is determined by comparison with the standard dye solution on a Coleman spectrophotometer, and the appropriate correction is made to the injected amount. This correction is of the order of 0.1 to 1.0 per cent.

Table I shows the amounts of dye remaining in a 1.5 ml. pipette after each of 23 intravenous injections of T-1824 in man. The mean value is 0.68 ± 0.07 per cent. A satisfactory correction of the injected amount can be made without measurement of the residual dye by simply subtracting this mean value.

DISCUSSION AND SUMMARY

Although a smooth, uninterrupted injection of an accurate amount of dye into the antecubital vein is one of the prerequisites in order to obtain good dye curves, other factors such as proper position of the arm and intravenous needle, suitable amount of dye according to the body weight of the subject, and free flow of blood through the brachial artery are equally important in the accurate determination of cardiac output by the dye dilution method.[2, 3, 5]

The design of precision pipettes and their application to the determination of cardiac output in man by the dye dilution method are described. The mean value of 0.68, standard deviation ± 0.32 per cent is the correction factor associated with use of the described pipette.

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-Sixth Annual Meeting

Chicago, Ill., Oct. 30 and 31, 1953

ABSTRACTS

1. THE INFLUENCE OF RELATIVE HUMIDITY ON THE SURVIVAL OF VIRULENT TUBERCLE BACILLI DEPOSITED ON SURFACES

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THERE have been some demonstrations, through animal inoculation, of an irregular presence of pathogenic tubercle bacilli in the air or on surfaces of environments contaminated by open cases of tuberculosis. Although persistence of living tubercle bacilli for prolonged periods following initial environmental contamination is widely accepted, a differentiation between "freshly seeded" and long term residual contamination is not made. Both may be important in the epidemiology of tuberculosis. Factors contributing to the survival of tubercle bacilli in environments have not been evaluated. The purpose of this study is to quantitate survival of tubercle bacilli deposited on surfaces and maintained at constant temperature (72° F.) and various relative humidities.

Atmospheres which remained constant at five different relative humidities (16, 33, 52, 75, and 95 per cent) were secured through use of saturated solutions of various salts in small sealed desiccator jars. On sealing the jars, an equilibrium characteristic for each salt was established between water in air and solution. Measured volumes of suspensions of human tubercle bacilli (H37Rv) in water, broth, and saliva were deposited on glass slides and these placed in the sealed jars. At increasing intervals, slides were withdrawn and the tubercle bacilli resuspended for quantitative colony counts.

At relative humidities of 52 and 75 per cent, irrespective of the suspending vehicle, no viable bacilli were demonstrable eight days after initial deposit of inocula. In water suspension, organisms persisted in reduced numbers for only eight days at 16 and 95 per cent relative humidities, and at 33 per cent relative humidity viability was demonstrable only until the thirty-third day. In broth suspension, organisms failed to grow after eight days at 16 and 95 per cent relative humidity and after fourteen days at 33 per cent. In saliva suspension there was no growth after the eighth day at 95 per cent, after thirty-five days at 16 per cent, and after thirty-seven days at 33 per cent relative humidity. Daily sampling of slides seeded with suspensions in pleural fluid and maintained at 52 and 75 per cent relative humidity revealed that loss of viability was manifested very sharply within twenty-four hours; the viable population continued to decline with extreme rapidity. These findings are of interest in that the tubercle bacilli survived only for unexpectedly short periods.

2. RADIOCHROMATOGRAPHIC IDENTIFICATION OF THYROXIN IN AN ALPHA GLOBULIN FRACTION OF SERUM SEPARATED BY STARCH ZONE ELECTROPHORESIS

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(INTRODUCED BY OVID O. MEYER, M.D.)

Radioactive iodine was administered in therapeutic quantities to four patients with hyperthyroidism. The serum was analyzed by paper electrophoresis to determine the distribution of radioactivity. Localization was found to occur in two zones, one in the slower moving albumin component, and another in the alpha zone just ahead of alpha-2 globulin. The radioactivity in the alpha zone was found to reach a maximum at four days.

The serum at four days was analyzed using zone starch block electrophoresis. The distribution of the radioactivity was identical with that seen with paper electrophoresis. The protein of these two zones was eluted from the starch with physiological saline. Each eluate was extracted with N-butanol. That in the albumin fraction was not butanol extractible and is assumed not to contain thyroxin. The butanol extracts of the alpha-2 globulin fraction of the four patients were analyzed using paper chromatography and found to consist entirely of thyroxin.

The absence of triiodothyronine in this fraction four days after oral administration of I^{131} does not exclude its presence in serum, as this amino acid is known to disappear rapidly from serum following intravenous administration. It is of importance, however, that the amino acid identified was thyroxin at a time when the radioactivity of serum is at a maximum following oral administration of I^{131}.

3. EARLY RESULTS OF THE TREATMENT OF CHRONIC GRANULOCYTIC LEUKEMIA WITH INTRAVENOUS COLLOIDAL $GOLD^{198}$

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(INTRODUCED BY FRANK H. BETHELL, M.D.)

Six patients with previously untreated chronic granulocytic leukemia were given colloidal $gold^{198}$ intravenously and have been followed for periods of from three to eight months. Two additional patients with late treated granulocytic leukemia were also given this isotope. In the previously untreated patients initial injections of from 23 to 45.5 millicuries were administered. Three patients have had a second dose given at an interval of from two and one-half to five months. There were no untoward or toxic reactions except mild nausea. Less than 0.5 per cent of the isotope was excreted in the urine during the first week. Plasma radioactivity fell precipitously to a low level within the first half hour. Levels of radioactivity in the white and red cells were determined for one patient. It was found that the leukocytes contained somewhat more of the isotope than the plasma, whereas the red cells showed negligible radioactivity. External counting studies and "scintiscan" records verified the high concentration of the isotope in the liver with a significant but lesser concentration in the spleen.

General clinical improvement was definite in five of the six previously untreated patients. All six showed regression in the size of the liver and spleen. Hematologic studies showed a distinct fall in the total white count in all of the previously untreated patients with an associated increase in the maturity of the granulocyte picture. In five of these cases an improvement in the red cell values occurred after the maximal fall in leukocyte count. Liver function tests were abnormal in most of the group before treatment. Subsequent studies showed no uniform change. In two patients for whom data are available the total blood cholesterol increased from abnormally low levels and an elevated basal metabolic rate decreased following treatment. One patient with late previously treated leukemia showed no benefit from therapy. The other patient, who was in terminal condition when the isotope was given, died two days later. Autopsy studies, including tissue radioactivity assays and autoradiograms, were performed.

4. PRODUCTION OF MEGALOBLASTIC MARROW BY ADMINISTRATION OF CORTISONE IN APLASTIC ANEMIA, WITH SUBSEQUENT RESPONSE TO VITAMIN B_{12}; A RELATIONSHIP NOT PREVIOUSLY DESCRIBED

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An 18-month-old child has been under observation and treatment since the age of 4 months because of a severe anemia characterized by normal white cell and platelet values, decreased hemoglobin and red count, and almost complete absence of reticulocytes from the peripheral blood, and by a marked decrease in nucleated red cells in an otherwise normal bone marrow. The patient developed a reticulocytosis when cortisone was administered, without significant rise in red cell or hemoglobin values. The red cells appeared to become macrocytic, and a second marrow examination showed many megaloblasts. Administration of vitamin B_{12} and folic acid produced a second reticulocytosis with a subsequent rise in hemoglobin. Cortisone was discontinued and the patient reverted to pretreatment status despite continued administration of B_{12} and folic acid. Readministration of cortisone while continuing B_{12} and folic acid resulted in another reticulocyte response, to 21 per cent, and rise in red cell values. Splenectomy was performed, after which all therapy was discontinued; the anemia recurred. Readministration of cortisone appeared to have little effect on the peripheral count, but reinstitution of B_{12} therapy while continuing cortisone produced a reticulocytosis of 40 per cent and a return of hemoglobin to 13 grams. Combined therapy was continued for six months, following which cortisone was discontinued and the patient observed. The red cell and hemoglobin values have fallen somewhat, but the most recent observation suggests that the relapse may be only partial. The patient is continuing under observation.

This patient appears to have an aregenerative anemia limited to the red-cell series, characterized by severe diminution of nucleated red cells in the marrow. The anemia is refractory to folic acid and B_{12}, but under the influence of cortisone red-cell development occurs in the marrow to the megaloblastic level and no further, following which a splendid response to B_{12} is exhibited. This relationship between cortisone and red-cell development is not known to have been described previously.

5. STUDIES ON THE ANTIPYRETIC ACTION OF CORTISONE

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The mode of action of cortisone as an antipyretic has been studied in rabbits challenged with intravenous injections of bacterial pyrogens. With both Pyromen and dextran, the fever was found to be markedly suppressed when cortisone was administered in liberal amounts (25 mg. twice daily) for three days prior to the challenge. Although the cortisone effectively blocked the febrile response to both pyrogens, it failed to influence the transient but marked leukopenia which characteristically precedes the onset of fever.

The antipyretic action of the drug also was shown to bear no relation to the activity of the "serum factor" recently demonstrated by Farr, Grant, and others to be involved in the production of pyrogen-induced fever. In preliminary experiments with typhoid vaccine as the inciting pyrogen, the presence of "serum factor" activity in normal blood and its absence in the blood of pyrogen-tolerant rabbits was confirmed. Subsequently the blood of rabbits treated with antipyretically effective doses of cortisone was shown to contain just as much "serum factor" activity as that of normal rabbits. In addition, previous incubation of the pyrogen with "serum factor" failed to influence the antipyretic effect of the drug.

It is concluded from these findings that in suppressing pyrogen fever cortisone acts neither upon the leukopenic reaction nor upon the fever-accelerating "factor" of the serum. By exclusion it would appear that the drug must influence some later stage of the fever-producing process. The mechanisms involved in the later stages of the response to exogenous pyrogen remain undefined, and the need for determining whether they are related to the prefebrile leukopenia is emphasized.

6. THE RELATIONSHIP BETWEEN THE METABOLIC STATE OF TUBERCLE BACILLI AND THE UPTAKE OF C^{14} ISONIAZID

WILLIAM R. BARCLAY, M.D. (BY INVITATION), ROBERT H. EBERT, M.D., AND DIETER KOCH-WESER, M.D. (BY INVITATION), CHICAGO, ILL.

Tubercle bacilli which are sensitive to isonicotinic acid hydrazide (isoniazid) become radioactive and cease to multiply if incubated at 37° C. with C^{14}-labeled isoniazid. These organisms retain this radioactivity despite prolonged and repeated washings with normal saline. Tubercle bacilli which are resistant to isoniazid do not become radioactive and continue to multiply when they are incubated with C^{14}-labeled isoniazid. C^{14}-labeled nicotinic acid, nicotinamide, and isonicotinic acid do not inhibit the multiplication of tubercle bacilli, nor do they render the bacilli radioactive.

Bacilli which have been killed by exposure to formalin or by heating at 60° C. for thirty minutes do not become radioactive when incubated with C^{14}-labeled isoniazid. However, tubercle bacilli which have stopped growing, and no longer utilize oxygen after exposure to streptomycin, become radioactive when they are incubated with C^{14} isoniazid. Likewise, bacilli exposed to C^{14} isoniazid at 5° C. become strongly radioactive even though growth and normal metabolism are arrested at this temperature.

These experiments suggest that the inhibition of growth of tubercle bacilli by isoniazid is associated with absorption and retention of the drug by the inhibited organisms. Bacilli resistant to the effects of isoniazid do not

absorb and fix the drug within the bacterial cell. This absorption of isoniazid by susceptible organisms is apparently specific for isoniazid and does not occur with isonicotinic acid, nicotinic acid, or nicotinamide. Heat killed or formalin killed bacilli are not able to absorb and retain isoniazid but arrested organisms by virtue of previous exposure to streptomycin or by being maintained at 5° C. are able to absorb and retain isoniazid. This suggests that resting organisms in vivo may be affected by exposure to isoniazid.

7. THE RELATIONSHIP OF EXOPHTHALMOS TO VARIATIONS OF THYROID FUNCTION

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ST. LOUIS, MO.

In 1934, the writer reported that post-thyroidectomy increase in exophthalmos was characteristically associated with hypothyroid states, rather than with recurrent hyperthyroidism as then generally assumed. It was reported that the edema of the orbital fat and extraocular muscles was reversible by the administration of desiccated thyroid substance if the condition was of brief duration, but that it was irreversible if chronic lymphedema had been replaced by fibrosis of these tissues. Since 1936, serial observations with the Luedde Exophthalmometer have been made on all thyrotoxic patients with abnormal eye signs and, in recent years, on many patients without hyperthyroidism who required thyroidectomy. These data are being analyzed and a summary and the conclusions to be drawn from them will be presented, together with a summary of such other clinical and experimental data as bear on the behavior of the eyes in disturbances of thyroid function.

8. CREATINURIA IN MYOCARDIAL INFARCTION: A PRELIMINARY REPORT

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(INTRODUCED BY JAMES A. GREENE, M.D.)

The pattern of urinary creatine excretion has been studied in eight patients with acute myocardial infarctions and angina pectoris and is being studied in a comparable group of hospitalized "normals." Serial twenty-four hour urinary excretion has been determined for fourteen days following hospitalization. In the cases of acute myocardial infarction having diagnostic electrocardiographical changes, creatinuria increased significantly by the fifth post-infarction day. There was not a similar increased creatine excretion in the cases of angina pectoris or the control group.

The possible value of the procedure as an adjunct in the diagnosis of acute myocardial infarction will be discussed.

9. STUDIES ON THE POSSIBLE RELATIONSHIP OF HEMOLYSIS TO THE THERMOGENIC RESPONSE FOLLOWING INTRAVENOUS FAT

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Previous work by Creditor has suggested that increased red cell fragility which occurs during fat infusion may be related to the thermogenic response. The present study has been carried out to determine (a) whether hemolysis

may be involved in the fever reaction following intravenous fat emulsions, and (b) if hemolysis, per se, is thermogenic. Bile fistulas were produced in rabbits by cannulating the common bile duct with a polyethylene tube which was brought through the abdominal wall. Following an eighteen- to twenty-hour postoperative period of stabilization, hourly temperatures and bile collections were made for a three-hour control period, and then fat emulsion (10 per cent sesame oil—Abbott) 10 ml. per kilogram was given intravenously over a five-minute period. Total hourly bilirubin output was then determined using the method of Evelyn and Malloy, and correlated with temperature changes occurring in the three- to five-hour postinjection period. Since the gall bladder had been ligated during the surgical procedure, collection of all bile was assured. Although a mean maximal temperature rise of 1.4° F. was observed after 13 separate injections in eight rabbits, in no instance was there a corresponding increase in total bilirubin output. On the contrary, in each observation decreases ranging from 10 per cent to 40 per cent occurred, which could not be accounted for by a concomitant fall in the mean hourly volume of bile except in two instances. The cause of this reduction is not readily apparent, although a decrease in hepatic blood flow would seem a more likely explanation than either decreased bilirubin formation or increased storage of bilirubin by the liver.

Nine separate intravenous injections of sterile distilled water in four rabbits in doses ranging from 0.25 ml. per kilogram to 5.0 ml. per kilogram resulted in increases of bilirubin output from 25 per cent to 400 per cent with no appreciable corresponding temperature elevation. Likewise, in two rabbits given phenylhydrazine intravenously, 5 mg. per kilogram, hemolysis as indicated by a fourfold increase in total bilirubin output was observed for a period of forty hours with no thermogenic response.

Our studies indicate that the thermogenic reaction of this particular fat emulsion was not accompanied by hemolysis. Furthermore, it would appear from these initial observations that, under the conditions studied, osmotically and chemically produced hemolysis is nonthermogenic.

10. CHARACTERISTICS OF LEFT ATRIAL PRESSURE PULSE WAVES RECORDED AT THORACOTOMY FROM NORMAL HUMAN HEARTS AND FROM THOSE WITH MITRAL STENOSIS AND REGURGITATION

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Accurate characterization of left atrial pressure pulse waves is prerequisite to direct hemodynamic study of mitral disease. Therefore, at thoracotomy such waves have been recorded electronically through a catheter in 36 patients: 2 with normal hearts, 29 with predominant stenosis, and 5 with predominant regurgitation.

The normal pattern began with a gentle auricular contraction wave ("a"), starting near the peak of the electrocardiographic P wave. Superimposed on its downstroke, beginning about 0.04 second after the R peak, was a sharp and narrow mitral closure wave ("c"), followed by a trough associated with downward movement of the mitral valve ring during left ventricular systole. Beginning about 0.15 second after the R peak, there followed a gentle and broad atrial filling wave ("f"), which fell to the end-diastolic pressure level after opening the mitral valve.

In predominant mitral stenosis with sinus rhythm four patterns appeared: (1) normal configuration at supernormal mean pressure levels; (2) same with interpolation of a small regurgitant wave ("r") between "a" and "f"; (3) incorporation of "e" and "r" into a single larger wave; (4) fusion of "a," "r" and "f," with tendency to loss of "e."

Patients with predominant stenosis and auricular fibrillation had patterns similar to (3) and (4) without "a" waves. A fifth pattern of smoothly undulating single waves was found in this group. All of these complexes seemed to represent combinations of the "r" and "f" waves, with closure waves being absent or obscured.

Two patients with predominant regurgitation and normal rhythm showed a small "a" wave followed by a tall, smooth, fused "r-f" wave beginning shortly after the R peak. Tracings from three patients with predominant regurgitation and auricular fibrillation resembled patterns (4) and (5) above but without "a" waves.

Thus, it has been possible to categorize and describe left atrial pressure pulse waves from normal hearts and those with mitral disease. Efforts are being made to establish morphologic criteria for quantitating at thoracotomy the degree of regurgitation which should contraindicate manipulation of the mitral valve.

11. GLOSSOPHARYNGEAL BREATHING

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(INTRODUCED BY KENNETH G. KOHLSTAEDT, M.D.)

A few patients with chronic respiratory paralysis due to poliomyelitis develop a peculiar voluntary method of respiration, glossopharyngeal breathing or "frog breathing." Using the mouth, cheeks, tongue, pharynx, and larynx they are able to force air into the lungs. After having exerted the maximum effort possible with the thorax and diaphragm, the patient gulps air with an open glottis, closing it after each gulp, trapping the air in the lungs. After the lung capacity is reached, usually after 6 or 7 such gulps, the glottis is released and passive expiration follows.

The purpose of this study was to determine the physiologic efficiency of this method of breathing. It was performed upon a 25-year-old chronic poliomyelitis respiratory invalid.

On a rocking bed cycling at 20 per minute the patient had a 535 ml. tidal volume, and an arterial oxygen saturation range of 92.5 to 95.0 per cent.

The average tidal volume with normal thoracic respiration was 267 ml., giving a 4,000 ml. minute ventilation at an average rate of 15 per minute. After five minutes of "normal" breathing the arterial oxygen saturation fell to 71.4 per cent.

With glossopharyngeal breathing the patient had an average tidal volume of 780 ml.: 248 ml. with thoracic respiration and the remaining 532 ml. effected by 6 or 7 gulps each with an average volume of 86 ml.

Glossopharyngeal breathing, 11 cycles per minute, contributed 5,650 ml. of the total 8,580 ml. minute ventilation. The range of arterial oxygen saturation at varying periods up to 45 minutes was 91.5 to 92.1 per cent.

Intratracheal pressure was measured through the tracheotomy stoma with a water manometer. With the glottis closed, just prior to the first gulp,

the pressure became positive, 3.5 to 4.0 cm. water. Each succeeding gulp raised the pressure 1.0 to 1.2 cm. with an end intratracheal positive pressure of 10 to 12 cm. of water.

It is apparent from this study that glossopharyngeal breathing is a physiologically efficient voluntary type of respiration in this patient.

12. A POSTOPERATIVE SYNDROME FOLLOWING MITRAL VALVULOPLASTY

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(INTRODUCED BY EDWARD MASSIE, M.D.)

While following patients who had undergone mitral valvuloplasty it became apparent that a significant number were developing an unexpected complication not seen after other thoracic surgical procedures. This complication occurred in seven of 75 patients operated upon.

Symptoms began approximately three to four weeks following operation with the onset of general malaise. Four patients developed fever. Two of these also noted arthritis and had hot, swollen joints. All developed a tachycardia. There was a marked increase in the symptoms of congestive failure in four patients despite strict cardiac management. Patients were treated with bed rest and salicylates in addition to their previous medication. Symptoms gradually disappeared in a period of four to twelve weeks leaving no residuals.

Clinically, this symptom complex strongly suggested the occurrence of rheumatic activity. This is unproved at present. All patients were treated with penicillin from the day of operation to the onset of symptoms, making the presence of an unsuspected beta hemolytic streptococcal infection unlikely. No evidence of rheumatic activity was present at the time of operation. It is conceivable that trauma to the heart might in some way be related to the onset of this syndrome. Further studies are being pursued to clarify these problems.

It is important to recognize this syndrome following mitral valvuloplasty since the exacerbation of symptoms suggests that the operation has produced further cardiac disability. However, all patients responded to symptomatic treatment and recovered without sequelae. These patients subsequently do as well as the uncomplicated cases but require more time to reach a comparable state of improvement.

13. RELATION OF ACIDOSIS TO URINARY AMMONIA EXCRETION IN PATIENTS RECEIVING CATION EXCHANGE RESINS

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A cation-anion exchange resin mixture was administered to 16 adult patients with edema of noncardiac origin. Seven patients had severe chronic glomerulonephritis with a nephrotic syndrome and subnormal urea clearance

tests. Nine patients had cirrhosis of the liver with massive ascites and edema, and normal urea clearance tests. There was no nitrogen retention in either group.

Urine ammonia nitrogen excretion was determined before treatment and on the eighth day of resin administration. In the patients with cirrhosis there occurred a marked increase in urine ammonia per liter (55.1 to 172.8 meq ammonia N/L) and per twenty-four hours (104.9 to 238.4 meq ammonia N). No such increase in urine ammonia occurred in the patients with chronic glomerulonephritis in whom the concentration and total excretion actually fell slightly during resin administration (76.7 to 61.1 meq ammonia N/L) (105.9 to 83.1 meq ammonia N/24 hours).

One hundred fifty-six paired determinations of serum pH and CO_2 content were made. In general a parallel decline in serum pH and CO_2 content occurred in both groups during the administration of resin. However, the fall was greater in the patients with chronic glomerulonephritis (pH from 7.412 ± 0.047 to 7.321 ± 0.050 and CO_2 content from 25.87 ± 4.66 to 18.21 ± 4.39) than in those with cirrhosis (pH from 7.403 ± 0.036 to 7.350 ± 0.028 and CO_2 content from 25.77 ± 3.33 to 21.03 ± 2.08).

The degree of acidosis developing during the administration of a cation-anion exchange resin appears to be inversely related to the ability of the kidney to increase ammonia formation and excretion.

14. RELATION OF CREATININE COEFFICIENT TO LEANNESS-FATNESS IN MAN

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Early studies have shown that obese, healthy individuals had lower creatinine coefficients (milligram creatinine excreted/24 hr./kg. body weight) than did lean ones. Methods of quantitating obesity were relatively crude. The present data were gathered incidental to another study. One twenty-four hour urinary creatinine determination per week was made for nine to ten consecutive weeks on 78 soldiers aged 18 to 37 years during various activity, dietary, and climatic states. Conditions were comparable for each member of the group. During three of these weeks basal body weight and several body measurements were taken. "Per cent body fat" was calculated from three skinfold thicknesses according to the formulae of Brozek and Keys (Brit. J. Nutrition **5**: 194, 1951) and Rathbun and Pace (J. Biol. Chem. **158**: 667, 1945). We have previously noted that the ratio of height to abdominal girth reflects leanness-fatness (Federation Proc. **12**: 178, 1953). The correlation coefficients here were: between "per cent body fat" and height/girth, –0.863; between "per cent body fat" and creatinine coefficient, –0.643; and between height/girth and creatinine coefficient, 0.548. All of these correlations are highly significant. The first correlation is significantly better than the other two, and the second significantly better than the third. Thus, the creatinine coefficient, though a valid measure of obesity, is not as accurate as simpler anthropometric measures. In this series, 26.05 plus the creatinine coefficient times –0.782 gave an estimate of "per cent body fat" with a standard error of regression of 2.7 per cent. Similarly, 37.55 plus –1.410 times the height/girth ratio gave the "per cent body fat" with a standard error of regression of 1.8 per cent.

15. THE PULMONARY HEMODYNAMIC PATTERN IN PATIENTS WITH ATRIAL SEPTAL DEFECTS BEFORE AND AFTER CLOSURE

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(Introduced by Matthew Block, Ph.D., M.D.)

The development of pulmonary hypertension is the critical factor heralding the onset of deterioration in patients with atrial septal defects. Twenty patients evaluated by cardiac catheterization were divided into two groups in an attempt to investigate factors leading to the development of elevated pulmonary arterial pressure. Group I consisted of eleven patients with normal pulmonary artery pressures, the mean pulmonary artery pressure averaging 15 mm. Hg. These patients were essentially asymptomatic. Microscopic examination of lung tissue obtained by biopsy in two patients and at necropsy in one patient showed no abnormalities in the pulmonary vasculature. Group II was comprised of nine patients with pulmonary hypertension, the average mean pulmonary artery pressure being 43 mm. Hg. Seven patients in this group were symptomatic, one having progressed to a fatal termination. Histological studies of lung tissue obtained by biopsy in one patient showed medial hypertrophy of the pulmonary metarterioles. Autopsy material in the second patient showed marked alteration in the pulmonary vasculature.

The average age in each of these two groups was 19 years. The average pulmonary blood flow in Group I was 13.6 liters/min./M.^{2}, and in Group II, 9.8 liters/min./M.^{2}. The total pulmonary artery resistance was normal in Group I, averaging 124 dynes/sec./cm.$^{-5}$, and was elevated in Group II, the average being 352 dynes/sec./cm.$^{-5}$. Thus, no satisfactory criteria for the prediction of the development of pulmonary hypertension were apparent.

Complete closure of the atrial septal defect was accomplished in three patients under direct vision employing hypothermia. Postoperative evaluation of these patients revealed a normal oxygen saturation of the mixed venous blood within the pulmonary artery and no evidence of a left to right shunt at the atrial level.

The postoperative findings were particularly striking in one patient. The pulmonary arterial pressure decreased from 100/30 mm. Hg to 30/17 mm. Hg, the pulmonary flow from 8.9 liters/min./M.^{2} to 3.0 liters, and the pulmonary arteriolar resistance from 253 dynes/sec./cm.$^{-5}$ to 208 dynes/sec./cm.$^{-5}$.

It would appear that not only can pulmonary hypertension be prevented but existing pulmonary hypertension can be strikingly reduced by closure of an atrial septal defect.

16. AN INVESTIGATION OF THE ROLE OF THE PULMONARY ARTERIAL OXYGEN CONTENT IN THE GENESIS OF PULMONARY HYPERTENSION

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(Introduced by Gordon Meiklejohn, M.D.)

It has been repeatedly demonstrated that hypoxia and exercise uniformly produce an increase in pulmonary artery pressure in patients with pulmonary hypertension. Three groups of patients with pulmonary hypertension have

been evaluated by various methods in an attempt to elucidate the factors responsible for the elevation of pulmonary artery pressure under these conditions.

While an increase in the pulmonary blood flow with exercise may produce a pressure rise in the presence of an altered pulmonary vasculature it may be demonstrated that this factor is not the sole element responsible for the resultant pressure elevation. Thus, a group of seven patients, with a resting cardiac index of 2.46 liters and an average mean pulmonary artery pressure of 51 mm. Hg, revealed with exercise a significant increment in the mean pulmonary artery pressure to 74 mm. Hg, while the increment in cardiac index was insignificant, increasing to but 2.50 liters.

A rise in pulmonary venous pressure contributes to the rise in pulmonary artery pressure. However, a group of nine patients revealed significant increases in their arteriolar resistance under hypoxic conditions and with exercise. This indicated contributing elements occurring at a pre "capillary" level and eliminated this facet as a major factor.

The significant remaining factor is the cross sectional area of the pulmonary vascular bed. The rapidly fluctuating pressure levels induced by exercise and hypoxia suggest an alteration in the tonus of the smaller pulmonary arterioles. Factors influencing the caliber of these vessels were evaluated. The possibility of lowered oxygen tension effecting pulmonary arterial tonus via the medullary vasomotor centers or within the pulmonary alveoli was eliminated by the correlation of the pulmonary artery pressure elevation with the peripheral arterial oxygen saturation and the alveolar oxygen partial pressure.

The effect of the oxygen content of pulmonary arterial blood on the smaller pulmonary arterioles was investigated. All groups of patients revealed a rise in pulmonary arterial pressure with a decrease in the oxygen content of the pulmonary arterial blood. It is thus suggested that a primary factor in the production of pulmonary hypertension is the oxygen content of the blood in contact with the pulmonary arterioles.

17. THE EFFECT OF INJECTED HEPARIN ON THE ELECTROPHORESIS OF THE LIPOPROTEINS IN PATIENTS WITH HYPERCHOLESTEROLEMIA

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(INTRODUCED BY SLOAN J. WILSON, M.D.)

The case material for the present report consisted of four cases of hypercholesterolemia with primary diagnoses of diabetes mellitus, familial xanthomatosis, lipoid nephrosis, and chronic biliary cirrhosis, and a normal control.

Each patient was given 50 to 100 mg. of heparin intravenously and a blood specimen obtained before and one hour after injection. Each serum was applied to several layers of Whatman No. 3 filter paper strips, saturated with barbital buffer at pH 8.6 and ionic strength 0.1 and subjected to electrophoresis for twenty hours between glass plates according to the technique of Kunkel and Tiselius. The strips were then cut at intervals of one centimeter and extracted for eight hours with hot ether-alcohol and the cholesterol and phospholipid determined for each strip. The protein pattern was determined from the same sera on a separate strip treated in the same manner.

The total cholesterol and phospholipid concentrations were found by integrating the values found on each small strip and the values found after heparin were in most every instance significantly greater than the value before heparin in the pathological cases, but were little changed in the control. The values for cholesterol and phospholipid determined from the whole serum showed little change or a decrease after heparin.

Striking changes in the electrophoretic curve of the cholesterol and phospholipid after heparin included a decrease in the sharpness and height of the lipid peak in the beta globulin region and an increase in the amount of the lipid in the alpha globulin region. These changes suggest the entrance of lipid into the protein bound phase from some other phase as well as a mean increase in the electrophoretic mobility of the lipoprotein. No significant change was noted in the pattern for the protein itself.

18. A STUDY OF EXPERIMENTAL SHOCK PRODUCED BY MENINGOCOCCUS TOXIN

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Bacterial infection is one of the causes of shock. Past studies indicate that this type of shock differs from traumatic shock in that there is no significant decrease in blood volume in most instances. Since it is difficult to perform hemodynamic studies on direly ill patients, bacterial shock was studied in dogs.

Detailed hemodynamic studies were performed on 30 dogs. Hypotension was produced by the intravenous administration of meningococcus toxin. Administration of cortisone (10 mg. per kilogram) prior to injection of toxin neither prevented nor accentuated the shock. The average decrease in blood pressure was 50 per cent and the average decrease in cardiac output was 48 per cent. The right atrial and pulmonary arterial pressures were decreased. The total peripheral resistance, plasma volume, total blood volume, and hematocrit readings showed no consistent changes.

The animals in shock were very sensitive to minor changes in blood volume. Removal of small amounts of blood (10 ml. per kilogram) caused marked further decline in cardiac output and blood pressure. Administration of dextran, 30 ml. per kg., invariably resulted in an increase in cardiac output usually to above the control levels with only a moderate increase in arterial pressure. Following the administration of dextran, the total peripheral resistance fell markedly. The animals in shock became relatively insensitive to the effect of norepinephrine on the arterial pressure, but if large doses were used the blood pressure could be raised above shock levels. The rise in blood pressure from norepinephrine was chiefly the result of a marked increase in peripheral resistance with only a small rise in cardiac output.

It is concluded that shock produced by meningococcus toxin in dogs is the result of peripheral vasodilatation. These studies suggest that both norepinephrine and a plasma expander are indicated in clinical therapy. The primary effect of norepinephrine is on the arterial pressure, whereas the effect of a plasma expander is on the cardiac output.

19. CHANGES IN COMPOSITION OF PNEUMOPERITONEUM AIR IN HUMAN SUBJECTS FOLLOWING INJECTION OF AMBIENT AIR

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(Introduced by C. L. Spurr, M.D.)

This is a study of the chemical composition of pneumoperitoneum air and of the rate of change following injection of ambient air. Thirty-two male patients, 25 to 67 years of age, 29 with pulmonary tuberculosis and 3 with emphysema, were studied. Gas analyses were done on 11 patients by the Van Slyke method and on 21 patients by the Haldane technique. The more accurate Haldane data are presented. No significant difference was noted between the Van Slyke and Haldane values. Average refills were 1,050 ml. at one week and 1,200 ml. at two weeks. Specimens one and two weeks following refill showed a mean composition as follows: CO_2, 6.08 per cent, σ ±0.093; oxygen, 4.90 per cent, σ ±0.42; nitrogen, 88.95 per cent, σ ±4.72. The preinjection composition of gas was similar in the groups receiving refills at one and two weeks. In six patients, repeated analyses over a two-month period showed marked stability of composition. These values were altered by the addition of ambient air, but reverted at differing rates to their initial values. Initial pneumoperitoneum with 1,000 ml. of ambient air showed a significant increase in CO_2 within one minute (0.31 per cent), reaching 1.88 per cent in ten minutes, 2.96 per cent in twenty minutes, and 5.82 per cent in twenty-four hours. Little change in the oxygen composition was noted during the first twenty minutes (19.85 per cent), but significant change was found at twenty-four hours (6.17 per cent) and at one week (3.83 per cent). It is felt that the rate of change of carbon dioxide concentration is sufficient in magnitude and rapidity to be important in practical therapeusis.

20. THE RENAL EXCRETION OF I^{131}: SIMULTANEOUS DETERMINATIONS OF I^{131} AND INULIN CLEARANCES IN VARYING STATES OF THYROID AND RENAL FUNCTION

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(Introduced by Joseph H. Holmes)

The present study represents an attempt to define the basic physiologic processes involved in the renal excretion of I^{131} in varying states of renal and thyroid function. Although the rate of renal excretion of I^{131} has been widely investigated as an index of thyroid function, little information is available regarding the fundamental processes of the renal excretion of iodide. Previous measurements of I^{131} clearance have been performed using single injection techniques, and the values obtained apparently have not been corrected for standard body surface area. In addition these values do not appear to have been related to other renal functions.

The clearance of I^{131} ($C_I{}^{131}$) has been measured simultaneously with inulin clearance (C_{in}) using standard continuous infusion techniques. A calculated dose of I^{131} (tracer level) was administered intravenously in the inulin-PAH priming solution, and plasma levels were maintained by the constant infusion of an I^{131}-inulin-PAH sustaining solution. Supplementary information was obtained from corresponding measurements of Na, K, and Cl in plasma and urine.

It was observed that C_I^{131} was roughly proportional to C_{in} in all groups studied despite a wide range of filtration rates; the higher C_I^{131} values corresponded to the higher glomerular filtration rates and the lower I^{131} clearances to the lower filtration rates. Thus the hyperthyroid cases, which tended to have high filtration rates, exhibited high iodide clearances, while the lowest I^{131} and inulin clearances to date have been observed in cases of myxedema and renal disease.

Comparison of percentage filtered load excreted $\left(\frac{C_I^{131}}{C_{in}}\right)$ with C_{in} reveals a decreasing $\frac{C_I^{131}}{C_{in}}$ with an increasing C_{in}. This has been tentatively interpreted as suggesting a regulatory role of the kidney with respect to iodine economy.

Theoretical concepts of the role of the renal tubules in the excretion of inorganic iodide will be discussed in the light of the foregoing data and in relationship to the renal excretion of other electrolytes.

21. RIGHT AURICULOVENTRICULAR PRESSURE GRADIENTS WITH SPECIAL REFERENCE TO TRICUSPID STENOSIS

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(Introduced by Harold Feil, M.D.)

Pressure curves obtained via right heart catheterization permit more accurate analysis of hemodynamic events when the auricular and ventricular curves are exactly superimposed. This can be done by a simple photographic method. In this manner the pressure changes in the two chambers can be correlated and instantaneous variations in the auriculoventricular pressure gradient measured. Normal and abnormal relationships have long since been elicited in dogs, using rigid cannulae (Wiggers). Clinically, in patients with acquired heart disease, certain characteristic patterns can be recognized: (1) chronic constrictive pericarditis—slight elevation of ventricular systolic pressure, diastolic "dip," and markedly elevated end diastolic pressure; high auricular pressure with abrupt dip following opening of A-V valve and following onset of ventricular systole; (2) right ventricular failure—elevated systolic and diastolic ventricular pressures, elevated auricular pressure with superimposition of A-V pressures during diastole; (3) tricuspid insufficiency—marked rise of auricular pressure with ventricular systole ("ventricularization" of auricle); with onset of diastole both pressures fall and then rise together; (4) tricuspid stenosis—high pressure gradient across A-V valve maintained throughout diastole. (In chronic constrictive pericarditis the ventricle fills suddenly and early in diastole, whereas in tricuspid stenosis, ventricular filling is slow.)

In a 38-year-old man undergoing cardiac catheterization prior to mitral valve surgery, the typical pressure gradients of tricuspid stenosis were elicited (in addition to elevated pulmonary capillary and pulmonary artery pressures). Valvulotomy of the mitral and then of the tricuspid valve was performed at one operation. (In bilateral valvulotomy the mitral valve must be operated upon first.) Catheterization studies four weeks postoperatively revealed a remarkable reduction of the pressure gradients toward normal, associated with dramatic clinical improvement. (We know of no other instance in which

bilateral valvulotomy was performed.) In order to confirm the clinical studies, tricuspid stenosis was produced experimentally in open-chest dogs and similar hemodynamic changes were observed.

Since tricuspid stenosis is now amenable to surgery and since approximately 14 per cent of all patients with mitral stenosis have definite tricuspid stenosis, the clinical recognition of this lesion becomes important.

22. POSSIBLE USE OF SERUM POOLS AS AN INDEX OF THE LEVEL OF IMMUNITY OF A METROPOLITAN POPULATION AGAINST INFLUENZA VIRUS

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Recent development of influenza virus vaccines offering promise of long-lasting immunity increases the importance of measures which might serve as an index of resistance of individuals and the general population to these viruses.

At the last meeting of this Society, we reported changes in antibody levels in serum pools collected weekly in St. Louis during the mild 1952 outbreak of Type B influenza. Our observations, now covering a period of more than two years, show a very decided increase in antihemagglutinins against Type A prime influenza viruses in serum pools during and following the influenza epidemic of January, 1953. Therefore, we have demonstrated in serum pools elevations in antihemagglutinin levels following periods of exposure to specific virus types. The rise in antibodies, however, follows by about two weeks the clinically observed onset of the epidemic.

Besides a number of stock strains, a Type B virus isolated by Dr. Dorland Davis in the outbreak of 1952 and three strains of Type A prime virus isolated in our laboratories during the 1953 epidemic were studied.

A very low antihemagglutinin titer was found against the newly isolated 1952 Type B strain in the six-month period preceding the Type B outbreak of that year. Similarly, low titers of antihemagglutinins were found against the newly isolated A prime strains in the pooled sera in the period preceding the epidemic of 1953.

The peculiar serologic behavior of newly isolated strains undoubtedly enters into this finding. However, we also observed that the hemagglutinins of the F.S. 1-50 (Cuppet) strain of A prime virus isolated in 1950, which serologically is closely related to the 1953 strains, likewise was neutralized by the serum pools only in very low titer. This stock strain, available before the epidemic, showed antibody levels in the pre-epidemic serum pools comparable to the virus strains subsequently implicated in the epidemic.

The possibility is suggested that serum pools collected in the summer and early autumn and tested against a well-diversified group of influenza virus strains might serve as a guide in a community to the need of immunization against specific virus types.

23. THE MECHANICS OF VENTILATION IN PATIENTS WITH HEART DISEASE

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An analysis of the mechanics of ventilation is essential to the understanding of the mechanism of dyspnea in patients with heart disease. The pressures involved in ventilation of the lung can be expressed by the formula,

$$P_T = P_L + P_P + P_V$$

P_T is the intrathoracic pressure, P_L is the pressure resulting from the elastic forces of the lung, P_P is the pressure drop required to overcome the resistance to gas flow, and P_V is the pressure required to overcome tissue friction. P_L is always negative. P_V and P_P are negative on inspiration and positive on expiration.

Intraesophageal pressure was used as a measure of intrathoracic pressure. P_L and $(P_P + P_V)$ were determined by measurement of P_T under static and dynamic conditions, respectively. A pressure-volume diagram was constructed from measurements of P_L at various levels of lung inflation. A pressure-flow diagram was constructed from measurements of $(P_P + P_V)$ at various levels of respiratory flow.

Seventeen patients with heart disease were studied and compared with 16 normal subjects. An alteration in the elastic properties of the lungs of the patients was demonstrated as an increase in the amount of pressure (P_L) required to produce a 100 ml. change in lung volume. The pressure-volume curves for normal subjects and patients with heart disease were identical when P_L was plotted against lung inflation expressed as per cent of vital capacity.

An evaluation of the relative importance of P_P and P_V was made by comparing pressure-flow curves obtained with the patient breathing air with those obtained with his breathing an argon-oxygen mixture. P_V was found to be a negligible factor and all the resistance to movement of the lungs could be attributed to resistance to gas flow (P_P). Resistance to gas flow, P_P, was found to be increased in some of the patients.

Thus, an alteration of the elastic properties of the lungs occurs in heart failure which is closely related to the reduction in vital capacity and is an important factor in the mechanism of exertional dyspnea. Certain patients with heart failure also demonstrate an increase in resistance to respiratory flow.

24. SKIN TEST RESPONSES TO INSULIN AND SERUM ANTIHORMONAL ACTIVITY IN A CASE OF COMBINED INSULIN ALLERGY AND RESISTANCE

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A 3½-year-old male diabetic child, admitted to the St. Louis Children's Hospital in moderate acidosis, was considerably resistant to insulin during hospitalization. Concurrently, he exhibited marked local reactions at injection sites, conjunctival reddening, and nasal congestion following injections and a blood eosinophilia of 17 per cent.

Direct intradermal skin tests were performed with 0.2 ml. of a number of insulin preparations. Crystalline insulins of beef and pork origin were separately tested and each yielded distinct positive skin reactions, while control subjects yielded negative reactions. Beef muscle extract also provoked a positive skin reaction, while pork muscle extract did not. Because the positive pork insulin and negative pork muscle reaction indicated sensitivity to an allergen in the insulin preparation, further identification of the allergen was attempted.

Week old neutralized insulins in 1 and 10 μ per milliliter strengths, presumably free of insulin hormonal activity, nevertheless yielded positive skin tests. A neutral trypsin digested insulin, free of hyperglycemic factor, yielded negative tests. A solution of hyperglycemic factor alone at 25 γ per milliliter concentration yielded a positive skin test comparable to that of crystalline insulin. Exposure of commercial insulin to boiling water for 30 to 180 minutes had no effect upon the skin reactive material. Treatment of the child during his resistant phase with trypsin digested insulin was no more effective than with commercial insulin in reducing blood sugar.

Serum obtained at the height of resistance was tested for its capacity to prevent insulin-induced hypoglycemic convulsions in starved 13 to 18 gram mice. The mice were injected with 0.25 ml. of serum adjusted to 400 mg. per cent glucose concentration and containing 0.072 units of insulin, and were then incubated for ninety minutes at 37° C. to observe for convulsions. Although some anti-insulin effect was present in the patient's serum, this did not significantly exceed that of control normal or non-resistant diabetic serum.

The observations suggest that (1) allergy to insulin was directed against a contaminant rather than the hormone and resistance did not seem related to this contaminant, (2) the allergen in commercial insulin is heat stable, and (3) serum from normal as well as diabetic patients may exhibit anti-insulin properties in mouse convulsion tests.

25. CLINICAL EVALUATION OF 6-MERCAPTOPURINE IN THE TREATMENT OF LEUKEMIA

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(Introduced by Frank H. Bethell, M.D.)

A new antimetabolite, 6-Mercaptopurine (6MP), has been shown to cause temporary remissions in a substantial percentage of children and an occasional adult with acute leukemia. Although in bacteria, 6MP presumably interferes with the incorporation in nucleic acid of preformed purines, their nucleosides or nucleotides, studies in animals and in man have not as yet revealed any reversal of toxic or therapeutic effects by such compounds. In children little or no toxicity occurred at the usual therapeutic dosage of 2.5 mg. per kilogram daily orally, but prolonged treatment at this level in adults or at higher levels in children occasionally caused bone marrow depression or gastrointestinal disturbances. Remissions lasting two to six months occurred after three to eight weeks of therapy in both high and low count leukemias, and in those resistant to cortisone and Amethopterin as well as in previously untreated cases.

Of 65 children with acute leukemia, clinical and hematologic remissions were achieved in 26 patients, partial remissions in 11, and 28 were considered

failures. Of the 40 adults, 5 aged 15, 21, 21, 35, and 52 years, respectively, had clinical and hematologic remissions and another 6 showed considerable clinical benefit with some improvement of marrow and peripheral blood. Twenty-nine were considered failures. Of the failures, however, 8 children and 10 adults had had only one to six days of therapy.

Remissions were achieved in all of 8 patients in the early stages of chronic myelocytic leukemia. Two of these have now been treated on maintenance therapy with the drug for nine and eleven months, respectively. When therapy was temporarily discontinued, relapses occurred in approximately four weeks, but further remissions were obtained with resumption of the drug. In one in the late stage with generalized lymphadenopathy, and in two in the terminal acute stage of the disease, temporary remissions were achieved.

In the acute leukemias, resistance to 6MP seemed to develop somewhat more rapidly than to Amethopterin, but there did not appear to be cross resistance between 6MP and cortisone or Amethopterin. In hopes of delaying the development of this resistance, combinations of 6MP with various other chemotherapeutic agents are under study.

26. THE EFFECT OF PROBENECID ON THE TUBERCULOSTATIC POTENCY OF PAS

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(Introduced by H. W. Schmidt, M.D.)

It has been demonstrated that probenecid alters the metabolism or excretion, or both, of para-aminosalicylic acid (PAS) so as to increase the concentration of free PAS in the serum. However, there have been no reports of clinical studies comparing the antituberculosis effects of PAS alone and in combination with probenecid. In fact, such a clinical study would be very difficult. For this reason we have studied this problem in experimental tuberculosis in guinea pigs.

The effect of premedication with probenecid on the concentration of free PAS in the serum of guinea pigs was studied first. It was found that premedication with 2 per cent probenecid in the diet significantly increased the concentration of free PAS in the serum of guinea pigs following a single dose of 400 mg. of sodium PAS given by intubation.

The effect of probenecid on the antituberculosis activity of PAS was studied next. Forty-six guinea pigs were each inoculated with 0.1 mg. of tubercle bacilli. On the twenty-first day 6 animals were killed and were found to have typical tuberculosis lesions. The remaining 40 animals were divided into four groups of 10 each. One group served as untreated controls, the second was given 2 per cent probenecid in the diet, the third was given 100 mg. of sodium PAS daily by intubation, and the fourth group was given both 2 per cent probenecid in the diet and 100 mg. sodium PAS daily by intubation. After sixty-two days of treatment the surviving animals were killed and examined for evidence of tuberculosis. The untreated controls and the animals given probenecid only had extensive tuberculosis. The animals which were given sodium PAS alone were found to have active disease but less than the first two groups. In contrast, the animals which were given both probenecid and sodium PAS had only slight evidence of tuberculosis, indicating that the combination was more effective than sodium PAS alone.

The clinical implications of this experiment are clear. It seems reasonable to predict that the concurrent administration of probenecid along with PAS would enhance the therapeutic effect of PAS in tuberculosis or permit the use of a smaller dose of PAS without loss of efficacy. Unless PAS is replaced by other antituberculosis drugs a clinical study of this problem should be made.

27. CARDIOVASCULAR FUNCTION IN PATIENTS WITH HEALED MYOCARDIAL INFARCTION

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The possibility that patients with healed myocardial infarction may have a latent circulatory defect is an important practical point. Those who develop angina pectoris or cardiac failure on recovery from the acute episode must, of necessity, limit their activity, but those who are asymptomatic often are able to resume very active lives. It has often been thought, however, that the activity of such patients should nevertheless be limited. In order to test the validity of this practice, hemodynamic studies were carried out on 9 patients of this type and, for comparison, a group of 12 normal subjects of comparable age was also tested. Cardiac output and mean circulation time were determined by the dye dilution technique before and during (not after) exactly 10 minutes on a treadmill set at 3 m.p.h. and 5 per cent grade. Intraarterial blood pressure recordings were also made during the same phases of the test. There was no statistically significant difference in the mean resting cardiac outputs for the 2 groups (5.71 ± 1.68 liters per minute for patients and 6.26 ± 1.77 liters per minute for normal subjects). Response of cardiac output to exercise was also similar in both groups (10.38 ± 2.70, and 10.97 ± 1.57 liters per minute, respectively). This represents an increase in cardiac output of about 1.8 times the resting value in response to exercise of this type. Mean circulation time was within normal limits at rest in both groups and there was no significant difference between them in the decrease due to exercise. Finally the degree and sequence of changes in blood pressure during and after exercise failed to differentiate the normal from the abnormal.

It is concluded that, although the test used does not determine circulatory capacity, it fails to disclose evidence of circulatory disability at rest or during moderate exertion in the patients as compared with the normal subjects. The results provide no support for the old notion, now mostly discarded, that such patients should be forbidden to indulge in any but the most sedentary activity.

28. PRIMARY PULMONARY ARTERIOSCLEROSIS

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Primary pulmonary arteriosclerosis is defined by Brenner as idiopathic arteriosclerosis of the pulmonary arterial tree with marked right ventricular hypertrophy and has been considered to be a rare disease. We wish to report nine cases studied including symptomatology, physical findings, teleroentgenograms, electrocardiographic tracings, cardiac catheterization, and angiocardiographic observations.

The most prevalent symptom was the early onset of progressive shortness of breath noted in all of the cases. In addition, hemoptysis, syncope, dizziness, pleuritic chest pain, and terminal cyanosis were frequently encountered.

Young, asthenic-appearing females predominated, making seven of our nine cases. An enlarged right ventricle to physical examination was present in all of them. An accentuated second pulmonic sound was uniformly heard. Pulmonary systolic murmurs occurred in two-thirds and pulmonary diastolic murmurs in one-third of the patients. Clubbing of the nails was conspicuously absent as was suffusion of the conjunctivae.

Teleroentgenograms routinely revealed evidence of right ventricular hypertrophy and a prominent undivided pulmonary arterial segment. In some a pulmonary "hilar dance" was present. No evidence of left atrial hypertrophy was seen. Electrocardiographic tracings showed right ventricular hypertrophy in seven of nine cases.

Right heart catheterization studies revealed marked elevation of right ventricular and pulmonary arterial pressures and decreased cardiac output. Slight reduction in the arterial oxygen volume's percentage occurred in the more severe ones. In two instances angiocardiographic films were taken to attempt to rule out pulmonary arteriovenous aneurysms but they were not found to be present. However, they did demonstrate the presence of a pulmonary infarction.

The disease was noted to be progressive, with the frequent development of pulmonary infarctions, serous effusions, and right-sided cardiac decompensation. Four of this series were autopsied and all revealed marked right ventricular hypertrophy, pulmonary arterial dilatation, pulmonary arteriosclerosis in the small arteries and arterioles, with marked reduction in the size of the lumen. No evidence of systemic atherosclerosis was noted in any of them. Associated pulmonary infarctions were present in two.

29. BLOOD PLASMA CONCENTRATION OF REDUCING CORTICOIDS IN HUMAN SUBJECTS

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(INTRODUCED BY SMITH FREEMAN, M.D., PH.D.)

Methods thus far reported in the literature give a variety of values for the normal concentration of adrenal corticoids existing in the circulation. The procedures described are laborious and either do not eliminate all interfering substances or incompletely reflect the extent to which various corticoids exist in the circulation. It is believed that the present method is free of some of the objections indicated above. This procedure entails the following steps: (1) Ten milliliters of human plasma are extracted with methylene chloride and the extract is washed countercurrently with dilute sodium hydroxide and water. (2) The residue of the washed extract is chromatogramed on a short strip of paper and washed free of interfering substances with hexane and methanol, respectively. (3) Elution from the paper strip nad color development by the tetrazolium blue method are carried out simultaneously in a colorimeter tube. According to this procedure the normal range for 26 adult subjects of both sexes was 24 to 52 μg per 100 ml. of plasma with a mean of 33.2 μg. Standard deviation, 8.27. Compound F is used as a standard.

The "corticoid" concentration in the plasma varies more from person to person than do repeated determinations on a given individual under constant conditions. The recovery of compound F added to plasma was 85 ± 5 per cent. Duplicate determinations on the same plasma agree closely. One patient with a diagnosis of adrenal insufficiency had 7 μg/100 ml. plasma.

Two patients with pituitary insufficiency had 12 and 15 μg/100 ml. plasma, respectively. ACTH infusion caused a rapid increase in the "corticoid" content of the circulation in normal subjects.

30. A COMPARISON OF STEROIDAL EXCRETION AND METABOLIC EFFECTS INDUCED IN MAN BY STRESS AND BY ACTH

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Extensive metabolic studies have been carried out upon four normal young men for the following purposes: (1) to compare the metabolic consequences of different stressor agents; (2) to determine the metabolic changes occurring in the same subjects when 2.5 mg. of ACTH are infused intravenously over a twelve-hour period in the absence of stress; (3) to note the metabolic modifications which take place when both the stressor agent and ACTH are administered together; and (4) to attempt to evaluate the role of adrenal cortical hormone in man's response to stress.

ACTH alone (2.5 units) produces a sharp increase in urinary 17-hydroxycorticoids and results in the well-known metabolic consequences. Stress, produced by either of two different agents (typhoid antigen and Pyromen) activates the ACTH-adrenal mechanism and results in a comparable increase in urinary 17-hydroxycorticoids. Despite this the metabolic consequences of stress thus induced are entirely different from each other and depend upon the stressor agent employed. Further, the classical metabolic pattern produced by ACTH in the absence of stress is not duplicated when the metabolic response is elicited by a stressor agent.

It is suggested that the concept of the stress reaction be enlarged to include specific responses which are dependent upon the character of the stressor agent, as well as the nonspecific activation of the ACTH-adrenal mechanism which occurs in the presence of stress; that such specific reactions against specific damage constitute defense against a specific stressor; and that adrenal cortical hormone plays a homeokinetogenic role in that it harbors the potentiality for releasing variable, multitudinous, and fundamentally independent forces which are marshalled in response to specific injury for the purpose of returning the affected systems to a state of homeostasis.

As a result of these studies Ingle's concept of the "permissive" activity of adrenal cortical hormone receives further support.

On the basis of the data a number of more specific points have been raised for further consideration. Among them is the possibility that the increased need for cortical hormone under conditions of stress is concerned more with the requirement of a higher concentration of the hormone in tissue fluids than with a process which degrades increased quantities of it.

31. THE HEMODYNAMIC, METABOLIC, AND ELECTROLYTE RESPONSES OF THE HUMAN HYPERTENSIVE KIDNEY TO THE INTRAVENOUS ADMINISTRATION OF SODIUM p-SULFAMYL BENZOATE

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(INTRODUCED BY WILLIAM S. MIDDLETON, M.D.)

This study was performed on seven fasting hypertensive patients utilizing standard clearance techniques and right renal vein catheterization before and after the intravenous administration of 2 Gm. of Sodium p-Sulfamyl Benzoate.

Within 15 minutes, and persisting for the 60 minutes of observation, following the injection of this agent statistically significant increases in the clearances of sodium (+160 to +2200 per cent) and C_{NA}/C_I ratios (+185 to +2700 per cent) were noted in all patients. These were associated with significant enhancement of the urinary pH, the clearances of potassium and the C_K/C_I ratios such, that in one patient, the latter proportion equaled 1.34. Chloride clearances and ratios showed greater variability, increasing in four, decreasing in two, and associated with no change in an additional patient.

Despite these marked alterations in electrolyte excretion no significant changes were noted in mean arterial blood pressure, urine flow, renal hemodynamics, or renal oxygen consumption (calculated as the product of total renal blood flow and arteriorenal venous oxygen difference). Simultaneous studies of cardiac output (direct Fick) in five patients showed changes varying from +24 to −37 per cent.

These results demonstrate the ability of Sodium p-Sulfamyl Benzoate to enhance the excretions of sodium and potassium by a specific blockade of renal tubular carbonic-anhydrase systems. In addition, these marked alterations, coupled with an unchanged renal oxygen consumption, fortify the concept that renal oxygen utilization is primarily directed toward the maintenance of the functional integrity of the kidney and to a much lesser extent toward the performance of external work.

32. CARDIOVASCULAR, CEREBRAL, AND RENAL HEMODYNAMIC AND METABOLIC ADJUSTMENTS TO 1-HYDRAZINOPHTHALAZINE IN ESSENTIAL HYPERTENSION

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Measurements of cardiac output (direct Fick), cerebral hemodynamics and metabolism (nitrous oxide method), and renal hemodynamics and metabolism (right renal vein catheterization) were made in twenty fasting hypertensive patients of Grades II to III severity before and thirty to sixty minutes after intravenous 1-Hydrazinophthalazine. Results are expressed in per cent change from control observations.

Cardiac Output (14 patients; 0.20-0.55 mg./kg.): A significant reduction in MABP occurred (27 per cent) accompanied by a decrease in total peripheral

resistance (41 per cent) and total pulmonary resistance (36 per cent). Cardiac output increased from 6.58 to 8.40 L./min. (28 per cent). Left ventricular work and mean pulmonary artery pressure were essentially unchanged.

Simultaneous Cerebral Flow and Cardiac Output (6 patients; 0.20-0.25 mg./kg.): MABP fell 29 per cent, while cardiac output and cerebral blood flow increased 24 per cent and 28 per cent, respectively. Total peripheral resistance decreased 38 per cent and cerebral vascular resistance 39 per cent.

Renal Circulation and Metabolism (6 patients; 0.25 mg./kg.): MABP fell 17 per cent, renal vascular resistance was reduced 38 per cent, and renal blood flow increased 55 per cent.

Oxygen consumption of the body, brain, and kidney remained unchanged at the time of significant increases in systemic, cerebral, and renal blood flow. There was a rise in oxygen content of the mixed (MPA), internal jugular, and renal venous blood with a corresponding decrease in oxygen extraction. Carbon dioxide content was reduced at the time of the second flow determination.

These studies indicate that the reduction in MABP following intravenous 1-Hydrazinophthalazine in hypertensive subjects is the result of a greater change in total peripheral resistance (–41 per cent) than cardiac output (+28 per cent). Systemic, cerebral, and renal vascular resistance decreased proportionately. Changes in systemic and cerebral flow were comparable. The per cent increase in renal blood flow (+55 per cent) exceeded the change in cerebral flow (+28 per cent) during a reduction in MABP from 145-120 mm. Hg and 153-109 mm. Hg, respectively.

33. AN APPLICATION OF EPIDEMIOLOGIC PRINCIPLES TO A STUDY OF THE PREVENTION OF INFLUENZA BY VACCINATION

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The protective effect of influenza virus vaccines has usually been measured by comparing the incidence of influenza in vaccinated and unvaccinated segments of a study group. The rate of infection in the unvaccinated fraction of that population serves as an index of the immediate risk of influenza. However, the presence of a large proportion of susceptibles in a group may permit infection to be established within that population. In these circumstances the full efficiency of immunization is not utilized. Vaccination of all individuals in a group provides maximal protection, since the proportion of susceptible individuals is thereby reduced to a minimum. In the present study the incidence of influenza in totally vaccinated and unvaccinated populations was compared. Sampson Air Force Base provided excellent facilities for this purpose. Recruits arriving at Sampson were assigned alternately to either of two training units. The training units were separated geographically, and contact between them was minimized. All of the recruits in one unit received influenza virus vaccine on the second or third day of processing. The other unit served as a control. Two experimental vaccines were employed. Each contained 700 CCA units of virus per ml. in saline. An outbreak of influenza A-prime occurred at Sampson early in January, 1953. Under the conditions of this study, the risk of noninfluenzal upper respiratory infection was approximately equal in both training units. The distribution of cases of influenza was proportionate among recruits housed in geographical subdivisions of each unit. Therefore, it seemed valid to attribute the difference in attack rates in

these two groups to immunization. It was demonstrated that the plan of immunization and the vaccines used gave a high degree of protection against influenza. Vaccination caused an excellent rise in antibody against the strain of virus isolated locally during the epidemic. In addition, the study provided an opportunity to demonstrate the value of sampling procedures and to compare the efficiency of compliment fixation and hemagglutination-inhibition methods for serologic diagnosis.

34. COMPARISON OF SERUM PROTEIN BINDING OF THYROXIN AND TRIIODOTHYRONINE

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(INTRODUCED BY OVID O. MEYER, M.D.)

Despite recent evidence that triiodothyronine plays an important role in thyroid metabolism, it is present in serum in quantities considerably less than thyroxin. Triiodothyronine when administered IV disappears more rapidly from the intravascular compartment than thyroxin. These two observations may be explained by the differential binding of triiodothyronine and thyroxin by serum proteins.

To study the in vitro binding of triiodothyronine and thyroxin by serum protein fraction these amino acids were added alone and in combination to serum which was then subjected to paper electrophoresis.

Radioactive thyroxin in physiologic quantities became bound to a specific alpha globulin (just ahead of α-2-globulin). Radioactive triiodothyronine was also bound by this protein but appeared to partially dissociate during the electrophoresis and trailed behind. In the presence of thyroxin, even in equal and physiologic quantities, triiodothyronine did not associate with this specific protein whereas triiodothyronine in quantities five times as great as thyroxin failed to interfere with the association of the thyroxin with α-2-globulin.

While triiodothyronine and thyroxin appear to associate with the same alpha-2-globulin, the thyroxin binding appears to be stronger. Thyroxin can replace triiodothyronine from this α-2-globulin.

35. OCCURRENCE OF AURICULAR FIBRILLATION IN IMMEDIATE POSTOPERATIVE PERIOD FOLLOWING MITRAL VALVULOTOMY

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(INTRODUCED BY SLOAN WILSON, M.D.)

We have selected sixty-eight patients for mitral valvulotomy. Twenty-eight of these patients maintained a normal sinus rhythm *prior* to the operation. Of these twenty-eight patients, eight began to be fibrillate within the first week following the operation. Six of the eight were reconverted to normal sinus rhythm.

Auricular biopsies from the entire series were examined in an effort to correlate the onset of the arrhythmia with pathological evidence of activity. No association was found.

No complications occurred as a result of the auricular fibrillation. The postoperative period was otherwise uneventful. Embolization was not recognized.

A possible cause for the arrhythmia is the edema and congestion secondary to the rather vigorous trauma to the auricle: cross clamping, stay sutures, purse string sutures, and amputation.

36. AN ATTEMPT TO ESTABLISH THE PRESENCE OF A HYPOCHOLESTEROLEMIC FACTOR IN THE RENAL BLOOD OF DOGS

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(Introduced by Sloan Wilson, M.D.)

Previously reported work by Miyazaki indicated that in rabbits renal venous serum from donor animals would markedly lower the serum cholesterol level in recipient rabbits. His work suggested that a kidney factor affecting the cholesterol level might be lacking in the hypercholesterolemia of lipoid nephrosis.

In an effort to extend this concept, we obtained renal venous blood from six dogs and measured the effect of this serum on the serum cholesterol of normal recipient animals.

Simultaneous renal and jugular blood were withdrawn and 2 ml. of serum per kilogram injected into respective recipient animals. A third animal, receiving no serum, served as a control. Serum cholesterol determinations were done on each of these three animals prior to injection, and at 1, 2, 3, 4, 12, and 24 hours. The cholesterol determinations were done by the method of Saifer, Abraham, and Kammerer.

A total of six such experiments were carried out. No significant changes in serum cholesterol occurred. We, therefore, conclude that, utilizing this experimental technique, we are unable to identify the presence of a hypocholesterolemic factor in the renal serum of dogs which affects the normal serum cholesterol of recipient dogs.

37. DYNAMICS OF THIRD HEART SOUND

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Using the calibrated technique for recording precordial vibrations developed by one of us, the method was applied to a case of calcific pericarditis with an unusually intense and diffused third sound. Following surgical removal of extensive portions of the pericardium, further vibration studies showed that the third heart sound had almost entirely disappeared. An analysis of our collection of 75 third heart sounds in the normal and in cardiac disease showed that it is a consistent part of the cardiac cycle in time of occurrence. Study of the vibration shapes of third heart sounds indicates that they are not due to eddies or flapping of valve leaflets, but resemble more the tensing of a membrane. These items are used to suggest that the third heart sound may be caused by the tensing of the pericardium at or near the completion of the filling phase.

38. A SYNDROME ELICITED BY PROLONGED ADMINISTRATION OF LARGE DOSES OF HYDRALAZINE

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Hydralazine in large doses (600 to 800 mg. daily) has been used over a long period (fifteen to thirty months) because of its sustained ameliorative effect in hypertensive disease. The major untoward sequel is a syndrome which appeared in 13 of 139 patients under treatment for at least three months. This, in its milder form, resembles early rheumatoid arthritis and, in its severe form, acute systemic lupus erythematosus.

The initial symptoms in the 13 patients were arthralgias in fingers and wrists, less commonly in other joints. Fusiform periarticular swelling of interphalangeal joints simulated rheumatoid arthritis in 8 patients in whom the symptoms progressed no further. These showed increased erythrocyte sedimentation rate, serum polysaccharide, alpha and gamma globulin, and decreased serum albumin; one was febrile, anemic, and leucopenic. The symptoms abated when hydralazine was discontinued.

Five of the 13 patients, either because their arthralgias were less severe, or their fortitude greater, continued to take the drug after the onset of arthralgia. These developed the severe form with fever (101 to 104° F.), prostration and anemia; 3 had polyserositis with abdominal and chest pain; one developed a severe erythematous reaction to ultraviolet irradiation and cutaneous changes resembling acute systemic lupus erythematosus; one had a diffuse, nonspecific "rheumatic" pneumonia. The serum albumin concentration was greatly decreased in all and the alpha and gamma globulin fractions increased. One patient developed a positive plasma L. E. test and another had L. E. cells in his bone marrow.

Withdrawal of hydralazine was followed by remission of the severe form in 1; the symptoms abated in another while treatment was continued. The patient with a primary pneumonic manifestation recovered after withdrawal of hydralazine and during administration of isonicotonic acid hydrazide. The latter was ineffective in 2 patients; these required vigorous corticosteroid therapy and 1 required maintenance steroidal treatment four months after withdrawal of hydralazine. Three of 8 patients with the milder and 2 with the severe form of the illness have continued hydralazine in doses of 150 to 300 mg. daily, taking aspirin for recurrent mild arthralgia.

The course of this hydralazine-induced illness differs from that of a typical drug hypersensitivity. The academic interest lies in its simulation of acute systemic lupus erythematosus.

39. THE INCIDENCE OF ARTERIOSCLEROSIS IN THE INTRAMURAL AS COMPARED TO EXTRAMURAL CORONARY ARTERIES

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In order to determine how frequently coronary arteries are covered during a part or all of their course by myocardium (intramural), 276 hearts were obtained from consecutive autopsies (the St. Louis City and the Barnes Hos-

pitals) and examined. The stimulus for this study came from a report by Geiringer (Geiringer, E.: The Relation of the Mural Coronary Artery to Arteriosclerosis, Am. Heart J. **41**: 359, 1951) that: (1) intramural segments of the left anterior descending branch of the left coronary artery had been found by macroscopic examination in 23 and by microscopic examination in 22 of 100 hearts; and (2) that atheromata occur only rarely in these mural stretches. He concluded that one of every five anterior descending coronary arteries in the human runs all or part of its course in the myocardium, and postulated that the myocardium protected these portions from developing atheromata.

Among the 276 human hearts in our series, only 15 instances of intramural coronary arteries were observed. All of these had one intramural portion with atheromata and narrowing of the lumen. Two were in the left circumflex branches and both were occluded by thrombi, one just proximal to its transition from extramural to intramural and the other in the intramural portion. Eleven of the remaining instances were found in the left anterior descending branches; only one involved the right coronary artery. Approximately one out of every twenty-four human hearts (5.4 per cent), therefore, had an intramural coronary for some portion of its proximal or midcourse.

Examinations of the arteries were made grossly, microscopically, and after microincineration. Severe, grade 4 involvement of the intramural segments (atheromata, fragmented or disintegrated elastic lumina, hyperplasia of intima, or with severe calcification) was present in eight of the specimens. Grade 2 atherosclerosis with intimal thickening occurred in six of the remainder. Only one intramural coronary artery had little or no degenerative change. In these observations there was no evidence to support the concept that covering of a coronary artery by heart muscle during all or part of its main course protects the artery from degenerative abnormalities.

40. THE COMPARATIVE EFFICACY OF VARIOUS MERCURIAL DIURETICS

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Twelve male patients with mild to severe congestive heart failure have been studied to (1) illustrate an approach for the evaluation of diuretic agents using controlled metabolic techniques and (2) compare the relative diuretic efficacy of Mercuhydrin, Thiomerin, Neohydrin, and SU 1775 (Ciba) when administered parenterally, as well as Neohydrin administered orally.

The patients were studied on the Metabolic Ward with a constant sodium, water, and caloric intake. An effort was made to wait until the patient was returned to an identical weight before the various diuretic agents were administered. The data suggest that, when this study was ideal in these respects, the changes in water, sodium, chloride, and potassium excretion, as well as weight, were more statistically significant.

The organic mercury thiol diuretic, SU 1775 (Ciba), was the most potent diuretic agent studied and was entirely free from local pain or other untoward effects which could be attributed to the drug per se. Four different dosages of SU 1775 were used (equivalent to 20, 40, 80, and 160 mg. of mercury). Three dosages of the other four agents were used (20, 40, and 80 mg. of mercury equivalents). At the 40 mg. of mercury equivalent dosage, the average per-

centage increase in sodium excretion was 368 for SU 1775, 277 for Neohydrin parenterally, 214 for Mercuhydrin, 191 for Thiomerin, and 109 for Neohydrin orally. The slope of the dosage response curve was greatest for SU 1775.

The use of controlled metabolic conditions to insure constant weight (and salt and water load) is suggested in evaluating diuretic agents.

41. THE EFFECT OF EXERCISE ON THE BLOOD PRESSURE AND PULSE RATE IN NORMAL SUBJECTS

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The effect of exercise on the blood pressure in normal human beings is still a subject of considerable controversy, partly because indirect sphygmomanometry yields inaccurate results during and immediately after exercise. In an effort to identify accurately the changes that take place under these conditions direct intra-arterial recording of blood pressure before, during, and after a fixed load of exercise was carried out in 19 young men (aged 18 to 39 years), 11 young women (aged 22 to 30 years), and 11 older men (aged 40 to 57 years). The recording system was mechanically damped so that a flat frequency response to 19 cycles per second was obtained. A motor driven treadmill, operating at 3 m.p.h. and set at 5 per cent grade, was used for the exercise. Measurements were made before the beginning, and after exactly ten minutes of exercise, but without stopping the treadmill. Continuous records were made during the first minute of recovery and for the first twenty seconds of the succeeding five minutes thereafter.

It was found that during exercise the systolic pressure rises significantly, the diastolic falls, and the mean remains approximately unchanged. In the first few seconds after stopping exercise, the systolic pressure fell precipitously in the male subjects but decreased more gradually in the women. In the older men, there was a significant "rebound" rise in the interval ten to forty seconds after cessation of exercise. The phenomenon involved all 3 pressure items. In all 3 groups, at the beginning of the sixth minute of recovery, the systolic pressure was close to or less than the resting value; the diastolic had exceeded the resting value and the mean was about the same as during rest.

The increase in pulse rate during exercise was similar in magnitude in all 3 groups. Pulse rates had not returned to resting values by the sixth minute of recovery.

If, as has been suggested, the response of the blood pressure to exercise is to be used for various diagnostic purposes, attention must be paid to differences in response that are probably attributable to age and sex factors rather than to disease.

42. THE EFFECT OF THORACO-CERVICAL VAGECTOMY ON CARDIOVASCULAR RESPONSE TO EXERCISE

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Cardiac rate in the human being is thought to be regulated by interaction of vagal and sympathetic impulses, the former presumably being dominant. Physiologic studies on a patient in whom about three-fourths of the vagal

pathways to the heart were surgically abolished are of interest in this connection. There was an immediate and permanent increase in resting pulse rate from about 74 beats per minute preoperatively to 120 afterward. Atropine caused no increase in postoperative rate. Resting cardiac output (dye dilution method) was normal four months after surgery. Response of cardiac output to a standard treadmill load was also normal, the increase being about 1.9 times. In normal subjects working at the same rate the increase is 1.7 to 1.9 times. The patient's increase in pulse rate during exercise, however, was significantly less than normal. The response of blood pressure to exercise was the same as that observed in the normal group.

It is concluded that absence of most, or all, of normal vagal pathways to the heart modifies, but does not abolish, the ability of the cardiovascular system to respond effectively to moderate exercise. The possibility that nonvagal influences may, under these circumstances, come into play is raised.

43. A NEW PHARMACOLOGIC PROPERTY OF QUINIDINE: PROTEIN ANABOLIC EFFECT

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The many biologic and therapeutic properties of quinidine and its levo isomer quinine are said to differ only quantitatively. Stereoisomerism about the secondary alcohol linkage between the quinoline and quinolidine rings does not alter plasmocidal activity significantly. However, qualitative distinction based on the isomerism of these two drugs may explain the observation that idiosyncrasy to quinine often extends to other levorotary cinchona alkaloids, but rarely to the dextrorotary compounds. As another qualitative difference between these two substances, therefore, it is of considerable interest to report our observation that quinidine sulfate, when administered by mouth, has a protein anabolic effect which is not shared by quinine sulfate when administered in the same manner.

A 67-year-old woman with nutritional and postmenopausal osteoporosis was placed on a metabolic balance regimen to evaluate the effects of long-acting testosterone esters. During the course of her study, spontaneous auricular fibrillation developed, and was treated successfully with quinidine sulfate. Subsequently, analysis of the balance data indicated that quinidine had induced a definite protein anabolic effect. The response to quinidine sulfate was studied a second time in this patient when she was not under the influence of a testosterone preparation, was in nitrogen equilibrium, and had no cardiac arrhythmia. The same dosage schedule was employed as previously. There was a prompt decrease in urinary nitrogen excretion and a gain in body weight. The phosphorus balance data showed a similar pattern. A repeat study with quinine sulfate in the same dosage revealed no significant anabolic effect.

The discovery of this unexpected quinidine sulfate effect may be important because: (a) it may augment our knowledge of the cardiovascular effects of quinidine, (b) it reveals a protein-anabolic action of an organic substance which differs chemically from compounds with known protein-anabolic activity and which may lead to other new agents with this activity, and (c) it may increase our understanding of the process of protein anabolism.

44. SERUM PROTEIN FRACTIONS IN RHEUMATOID ARTHRITIS

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In an initial group including one case of rheumatoid spondylitis, one of acute rheumatic fever, and six of rheumatoid arthritis, fibrinogen was determined by the method of Wu and serum proteins by the sodium sulfate fractionation procedure of Milne (J. Biol. Chem. **169**: 595, 1947). These methods are useful for detecting the frequent increase in fibrinogen and decrease in albumin. For a more extensive study of the serum protein pattern, its possible relationship to the stage of rheumatoid arthritis, and its response to treatment, filter paper electrophoresis was employed. The apparatus of Köiw, Wallenius, and Grönwall (Scandinav. J. Clin. & Lab. Investigation **4**: 47, 1952) was used, and the technique was similar to that described by these authors. However, the bromphenol blue bound by each serum protein fraction was eluted in one lot with 5 per cent sodium carbonate in 50 per cent methanol and determined spectrophotometrically. Reproducibility of the technique as well as the range of normal variation were determined by carrying out a large number of analyses on sera of 12 normal subjects and analyzing the data statistically to provide a basis for evaluating results in patients.

Detailed data are presented on normals and on 23 patients with rheumatoid arthritis in various stages of the disease. Of these patients, four showed normal protein partition and normal total protein; one had normal partition but low total protein; one showed slight elevation of beta globulin; seven had elevated figures for alpha-2 globulin; three demonstrated gamma globulin elevation; four showed elevation of gamma, beta, and alpha-2 globulins; two had elevation of alpha-2 and beta globulins; and one showed elevation of alpha-2 and gamma globulins. This series so far is too small to attempt any correlation between the various fractionation patterns and the stage or activity of the disease.

Three patients with rheumatoid arthritis had determinations repeated in the course of therapy, and showed a tendency to return toward normal partition patterns as clinical improvement occurred.

45. THE EFFECTIVENESS OF WATER AND OIL-SOLUBLE VITAMIN K PREPARATIONS IN CORRECTING HYPOPROTHROMBINEMIA

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Oil-soluble vitamin K_1 (Mephyton) is an effective antagonist to the hypoprothrombinemic effect of oral anticoagulants (both coumarin and indandione derivatives). Vitamin K_1 appears to be equally effective by the oral or intravenous route. Cessation of hemorrhage and safe prothrombin levels (40 per cent of normal concentration or more) were achieved in 40 of 44 trials within twenty hours. A single oral dose of vitamin K_1 varying from 5 to 25 mg. was effective in 15 of 17 trials. In 20 of 22 trials 50 mg. or less of intravenous K_1 produced satisfactory response in twenty hours or less. There

were four instances in which single dosages of this magnitude by either route of administration proved ineffective. Untoward complications to excessive dosages of vitamin K_1 have not occurred; minimal yet effective dosages of K_1 are desirable to avoid refractoriness, should use of oral anticoagulants be desirable later. In contrast to K_1 the water-soluble preparation of vitamin K (menadione bisulfite, Hykinone), is not consistently reliable as an antidote to the oral anticoagulants. With a dosage of 40 to 144 mg. of Hykinone safe prothrombin levels were attained in only 11 of 26 trials in twenty-four hours, with three more responding at the end of forty-eight hours.

Response to vitamin K cannot be predicted with certainty when hypoprothrombinemia is secondary to severe hepatic disease. Under these circumstances, an intravenous dose of 50 mg. of vitamin K_1 repeated three times in one week is recommended. There may be a gradual but progressive rise of the prothrombin to a final level which often falls short of the normal value. In correction of hypoprothrombinemia due to absorptive difficulties, 5 mg. of K_1 and 72 mg. of Hykinone have been observed to produce normal prothrombin levels in as little as four hours after intravenous injection.

46. CLINICAL AND LABORATORY EXPERIENCES WITH COMBINED PENICILLIN-DIHYDROSTREPTOMYCIN THERAPY FOR SUBACUTE STREPTOCOCCAL ENDOCARDITIS

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(INTRODUCED BY THOMAS J. DRY, M.D.)

During the past thirty-three months, 44 patients having streptococcal endocarditis have been treated. Penicillin-sensitive streptococci caused the endocarditis in 28 patients; the remaining 16 patients had enterococcal infections. The organisms of the first group were of the Viridans-Salivary group. Twenty-six strains were sensitive to 0.1 unit or less of penicillin; 2 others were inhibited by 0.2 and 0.5 units. Short-term therapy (two weeks) was employed for these patients utilizing 2,000,000 units of aqueous procaine penicillin G and 2 Gm. of dihydrostreptomycin daily, given in two equally divided doses every twelve hours. Twenty-three (82 per cent) of the patients have been cured of their infection for two months to twenty-eight months. No treatment failures or relapses have occurred in the living patients. The 5 deaths (18 per cent) resulted from congestive failure (3), cerebral embolism (1) and coronary embolism with myocardial infarction (1).

Bactericidal tests employing the patients' own serum and infecting organisms were performed in the last 5 treated patients. In each instance the antibiotic combination gave greater bactericidal effect than with penicillin alone.

The 16 enterococcal infections were caused by organisms resistant to 1 or more units of penicillin. Twelve patients are living (75 per cent) and 4 are dead (25 per cent). The deaths resulted from cerebral hemorrhage (2) and congestive failure (2). In 14 patients, combined therapy was given. In 11 of the 12 living patients, 6,000,000 to 50,000,000 units of aqueous crystalline penicillin was given by continuous intravenous drip for three to ten weeks. Dihydrostreptomycin sulfate, 2 Gm. per day, for two to five weeks, then 1 Gm. per day for the remainder of the period of treatment, was given. In the additional living patient combined therapy was given for ten days and then

penicillin alone (7,200,000 units per day) for sixty more days, when bactericidal tests indicated no greater killing effect with added streptomycin. Of the 12 living patients, relapses occurred in 5; all 5 patients were retreated successfully.

Combined antibiotic therapy for streptococcal endocarditis, results of bactericidal tests performed and antibiotic synergism will be discussed.

47. MECHANICAL HEART BLOOD PRESSURE PATTERNS WHICH PERMIT DIRECT VISUALIZATION OF THE MITRAL VALVE IN THE LIVING HEART

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(INTRODUCED BY WARREN B. COOKSEY, M.D.)

The normal arterial pressure pattern is due chiefly to ventricular ejection. Were it not for the energy imparted by the ventricles to the fluid vascular compartment, the "blood" pressure in the arteries would soon be dissipated. Therefore, by analyzing blood pressure patterns we learn more about the amount of work which the ventricles in the living animal are doing. Even though the blood pressure is affected by other factors, such as blood viscosity, peripheral resistance, blood vessel distensibility, etc., the primary source of energy is derived from the ventricles. Just as the arteries have pressure patterns characteristic of arteries, so do the ventricles exhibit their own characteristic pattern, as does any mechanical pump.

Our studies have been concerned with the temporary substitution of a mechanical pump to do the work of (1) the "right" heart, (2) the "left" heart, and (3) both right and left sides of the heart, so that the part of the heart which is bypassed can be opened for direct vision surgery.

By recording simultaneously the mechanical heart pump pressure pattern, the aortic pressure pattern, and the electrocardiogram, one can see when the aortic pressure pattern is completely replaced by the "mechanical heart" pressure pattern. When only the "mechanical heart" pressure pattern is seen in the aorta, then no blood is being ejected from the left ventricle, and the heart can be opened for mitral valve examination without hemorrhage.

The right ventricle has been substituted for in valvular pulmonary stenosis, and the left ventricle has been substituted for in mitral stenosis.

48. CORTISONE IN THE TREATMENT OF MYOCARDIAL INFARCTS: A PRELIMINARY REPORT

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(INTRODUCED BY WARREN B. COOKSEY, M.D.)

A series of experiments were undertaken to determine the effect of cortisone on the degree of residual fibrosis following the experimental production of myocardial infarcts in dogs.

The procedure was to ligate the anterior descending branch of the left coronary artery, using some of the animals for controls and treating the re-

mainder with cortisone. The treatment schedule for the various groups was as follows: (1) beginning immediately as the coronary artery was ligated and continuing daily for varying numbers of days, (2) delaying treatment for one to three days, (3) treatment prior to ligation without treatment following ligation.

Gross and microscopic examination, along with x-ray injection studies of the coronary vascular bed were made. Electrocardiograms were taken before, during, and after ligation of the coronary artery.

Studies indicated that in the treated animal there was a decrease in the immediate mortality rate; a decrease in the area of residual fibrosis of the infarcted area; and an increase in the vascularity of the heart as compared to the control animals.

With these findings a clinical program was set up to evaluate cortisone in the treatment of myocardial infarction in man and is being carried out.

49. POSSIBLE EFFECT OF GAMMA GLOBULIN PROPHYLAXIS ON SUSCEPTIBILITY TO REINFECTION

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Widespread use of gamma globulin to prevent or modify measles, infectious hepatitis, and poliomyelitis stimulates one to question the effect this measure might exert upon the immunity which results from these diseases. Measles confers solid lasting immunity and second attacks of this disease are extremely rare. Therefore, to determine whether infection attenuated by gamma globulin results in an immune state as effective as that which follows the natural diseases, it was only necessary to observe the presence or absence of second attacks of measles in a population in which modified measles was commonly seen. To obtain data on this problem cases of measles which occurred in a selected population of 85 families were studied. During the period of 1948-52, 84 cases of measles occurred among a total of 408 persons. Of these illnesses, 35 were attenuated by gamma globulin. In this group of 35 cases of modified measles, 2 episodes of second attacks were observed; an attack rate of 5.7 per cent. In addition, 2 other cases of measles were observed in this population group who had had authenticated measles modified by gamma globulin two and four years prior to their second attacks and before their entrance to the family study. Moreover, at least 6 cases of measles occurred in persons in whom this disease previously had been prevented clinically by gamma globulin. These data strongly suggest that attenuation by gamma globulin reduces the production of antibodies to the etiologic agent and therefore diminishes the degree of immunity usually conferred by the infectious process.

50. NONSPECIFIC PROTECTIVE EFFECT IN REPEATED PULMONARY INFECTIONS CAUSED BY GROUP A HEMOLYTIC STREPTOCOCCI

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In the course of developing methods employed in the study of certain effects of repeated streptococcal infections, rats were infected with a Group A strain and treated for seven days with procaine penicillin. One week after completion of therapy reinfection was attempted. The inoculum was introduced into the lung opposite that originally infected, and a serologically unrelated Group A strain was employed. Rats were sacrificed eighteen to twenty-four hours after the second infection was induced; impression smears were made from cut sections of the more recently infected lung. In such preparations, the infectious process appeared remarkably controlled when compared with sections taken from animals infected for the first time.

As a result of these unexpected observations, further studies were carried out to determine whether the mortality rate for rats, previously infected with streptococci and treated with penicillin, was indeed less than that for normal controls. In three such experiments 9 of 10, 4 of 12, and 19 of 21 reinfected animals survived, whereas respectively 2 of 7, 0 of 9, and 4 of 18 controls recovered. In other experiments, previously uninfected animals given identical courses of penicillin and infected seven days after completion of therapy exhibited mortality rates comparable to those of untreated normal rats. Likewise, animals inoculated with sterile gastric mucin and treated with penicillin were not protected against a subsequent streptococcal infection.

Additional studies along similar lines indicate that the protective effect observed in the experiments outlined is not solid, and can be obscured if the challenging infection is fulminating. Nevertheless, the results suggest that there is, under carefully controlled conditions, some degree of protection engendered by an initial streptococcal pulmonary infection against a subsequent one. Whether this phenomenon represents some measure of group specific immunity is at best speculative, especially since a large body of data supports the concept that immunity to Group A streptococcal infection is exclusively type-specific. Further investigations to evaluate this question are being pursued.

51. ACETATE METABOLISM OF BLOOD CHOLESTEROL SYNTHESIS

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A series of seven patients at the Argonne Cancer Research Hospital with limited life expectancies have been studied subsequent to the oral administration of 100 microcurie doses of C^{14} carboxyl-labeled acetate. The rates of appearance of C^{14} in the free cholesterol of plasma and red cells, and in the esterified fraction of blood cholesterol were measured. Total amounts of C^{14} in blood, urine, and expiratory CO_2 were also followed for several days, and the results will be presented. The specific activity (i.e., relative concentration of C^{14} in total cholesterol carbon) of the free and esterified plasma fractions reached a peak approximately three hours after acetate administration. The

specific activity of red-cell cholesterol approached that of plasma-free, and was usually slightly higher at the end of twenty-four hours; that of the plasma esterified fraction rose more slowly, but became essentially equal to that of plasma-free by forty-eight hours. After this time, the specific activities of all three fractions remained close together and, during the following eighteen days, decreased at rates in general agreement with a turnover time of about twelve days, as previously reported for serum cholesterol by London and Rittenburg.

These results are very similar to those previously obtained in several animal species, except that the time taken to reach the peak value for plasma-free cholesterol was shorter in dogs, but considerably longer in rabbits. Serial liver biopsy studies in dogs have shown that the liver-free cholesterol reached a peak specific activity value sooner than the plasma-free, and that the two rapidly approached the same value. It can be reasonably assumed that this takes place in human beings also. In all species, newly synthesized cholesterol appears in plasma much faster than it subsequently disappears, and its appearance must be due to a rapid rate of interchange of unesterified cholesterol molecules between liver and plasma. It is postulated that this is an important part of the mechanism of plasma-cholesterol homeostasis, i.e., changes in the plasma-cholesterol level rapidly result in changes in liver concentration which in turn affect the rate of hepatic cholesterol synthesis.

52. THE RESPONSE OF HYPERTENSIVE PATIENTS TO INTRAVENOUS 1,4(BIS 1,4-BENZODIOXAN-2-YL METHYL) PIPERAZINE (McN-181, DIBOZANE)

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(Introduced by Sloan J. Wilson, M.D.)

McN-181, pharmacologically a potent adrenergic blocking agent, was given intravenously to 35 patients with hypertensive diseases of varying types and severity.

A dose-to-tolerance method of administration was used. Electrocardiographic and occasional ballistocardiographic tracings were taken. The side effects were: light-headedness, drowsiness, nausea, vomiting, vertigo and nystagmus, singultus, confusion, increased irritability, preconvulsive clonic movements, paresthesias, dry mouth, perspiration, flatus, lacrimation, and palpitations. Weakness was a common manifestation.

Two patients with recent cerebral injury showed first a depression of the central nervous system. This was followed by a rise in blood pressure and then a preconvulsive state which subsided upon discontinuation of the infusion. This has been observed clinically in hypertensive patients with epilepsy and suggests mechanisms similar to those seen by van Harreveld in Leão's spreading-cortical-depression. This phenomenon precedes convulsive discharges in their animal experiments.

The blood pressure in the other 33 patients was lowered minimally to markedly with from 14 to 150 mg. of McN-181. One patient, suspected of pheochromocytoma, had a precipitous fall with the smallest dose. Although the heart rate was generally variable, a moderate decrease in rate was noted when the diastolic fell below 80 mm. The orthostatic response was seen in 54 per cent of patients. One patient developed sinus arrhythmia and bradycardia; another displayed a nodal rhythm. Occasional ventricular premature

contractions were precipitated in two other patients. Electrocardiographic strain patterns and abnormal BCG patterns were seen to revert to normal. In all cases the side effects produced were transient.

McN-181 is capable of lowering the blood pressure in most hypertensive patients while producing moderately severe side effects. The responses to its isomers and to the oral compound in chronic trials are currently being studied.

53. OBSERVATIONS ON THE COMPARATIVE EFFICACY OF ERYTHROMYCIN AND PENICILLIN IN STREPTOCOCCAL INFECTIONS

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(INTRODUCED BY CLAYTON G. LOOSLI, M.D.)

The recently discovered antibiotic, erythromycin, has been shown in preliminary clinical trials to have definite ameliorating effects on the course of many acute infectious diseases, including streptococcal infections. Substantial data have not as yet been accumulated on the comparative value of erythromycin versus penicillin therapy for acute streptococcal diseases. An opportunity was available at the Great Lakes, Illinois, and Bainbridge, Maryland, U. S. Naval Training Centers during the past winter to study a group of 322 patients with these diseases.

Naval personnel, mainly recruits, who were admitted to the hospital or to the regimental infirmary with a presumptive streptococcal infection were entered, in strict rotation, into one of three treatment regimens: erythromycin, procaine penicillin, or placebo. Varying dosages and durations of therapy with erythromycin were studied as well. Each patient was followed closely with daily clinical examinations and complete laboratory observations, including serology for streptococcal and influenzal antibodies.

From the information so collected, it then became feasible to compare the three therapeutic regimens on the basis of strictly objective criteria—the duration of fever, the duration of leukocytosis, the duration of positive cultures for beta hemolytic streptococci, the duration of the rash in scarlet fever, and the total duration of the illness. It was possible to obtain data on the ability of erythromycin to eradicate the streptococcus from the throat by comparing the results obtained with the various dosage schedules employed.

Briefly, the results appear to indicate that optimal dosages of erythromycin given orally and without regard to meals are fully as effective as aqueous procaine penicillin given intramuscularly for the same period of time, regardless of the criteria selected for comparison. It was also evident that suboptimal dosages or abbreviated durations of therapy resulted in failure of the drug to eradicate the streptococcus, as similarly shown previously with penicillin. Fewer reactions to antibiotic therapy were observed in the erythromycin-treated group. The inclusion of an untreated control group of patients allowed for statistically significant comparisons to be made on all criteria.

54. LIVER LESIONS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF POLYVINYL PYRROLIDONE (P.V.P.)

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Twenty-two patients free of disease of the liver, kidneys, or blood-forming organs were given an intravenous infusion of 1,000 ml. of normal saline containing 3.5 or 4.5 per cent of the plasma substitute polyvinyl pyrrolidone. Laboratory studies including liver and kidney function tests and needle biopsy of the liver were performed prior to the infusion and at varying intervals afterward during a period of sixteen months.

Histologic studies of the liver revealed the appearance of basophilic globular deposits ranging up to 50 microns in diameter which lay within Kupffer cells or free in the sinusoids and were occasionally accompanied by a mild inflammatory exudate. The deposit rarely appeared in the liver earlier than three months after the infusion but was encountered almost uniformly in specimens obtained after an interval of six months. Histochemical studies lend weight to the belief that the material deposited represents storage of PVP.

The liver function tests have remained normal with some exceptions, and there has been no detectable enlargement of the liver or spleen during the period of this study.

The histologic findings have indicated that PVP in tissue is relatively inert at least for short periods of time. Prolonged storage, however, and the minor inflammatory and fibroblastic reactions noted in several cases emphasize the need for continued study.

55. ALTERATIONS IN SERUM GLOBULINS, CHOLESTEROL, AND LIPOPROTEINS AS RELATED TO DIABETIC RETINITIS

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This study concerns the relationship between the levels of serum globulins, cholesterol, and lipoproteins and the development of retinitis in diabetes mellitus. Data were obtained from ninety-five patients who had developed diabetes mellitus before the age of 15 years and whose disease had been established for at least ten years. The duration of the disease was longer than fifteen years in 53 per cent and exceeded twenty years in 29 per cent.

Each patient's retinae were examined by the ophthalmologist of the research team who had no knowledge of other clinical findings or of the patient's diabetic history. His observations were recorded on a numerical scale extending from zero, designating normal retinae, through nine, indicating proliferative retinopathy. On the same day blood samples were collected for analysis. In each case the level of total plasma protein was determined and fractionation was done by electrophoresis in the Tiselius apparatus. Values for alpha-2

and beta globulin were recorded in percentage of total protein. In ninety-one cases the serum content of cholesterol and in forty-eight cases the content of lipoprotein of the S_f 12-20 molecular class were ascertained. (Lipoprotein determinations were done by Drs. Lauffer and Henig, University of Pittsburgh.)

Forty patients had retinitis evidenced by the presence of capillary aneurysm or more serious degeneration. Scattergrams were constructed by plotting the values for alpha-2 globulin, beta globulin, cholesterol, and lipoprotein (S_f 12-20) against the degree of retinitis. Analysis of these data shows no correlation between the presence of retinitis and the level of alpha-2 globulin in the plasma. In the instance of S_f 12-20 lipoprotein some correlation is found, but this is significant only at the 5 per cent level of confidence. Similar analysis of the relation between the degree of retinitis and the cholesterol level or the plasma content of beta globulin reveals a much better correlation, both being significant beyond the 1 per cent level. Of the two, the correlation for beta globulin is slightly better.

56. LATENT VIRAL INFECTIONS OF CELLS: AN EXPERIMENTAL APPROACH

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The ability of a number of viruses to remain dormant in host tissues for long periods without causing a manifest disease state is well recognized, as in the latent infections or carrier states seen in human infections with herpes and psittacosis viruses, which permit survival of virus in a population for long periods and also may account for the recrudescence of herpes simplex infections in man or psittacosis in birds. The nature of this alteration of host-parasite balance which results in recurrent disease is poorly understood.

An experimental approach to the problem of virus latency is presented here, using psittacosis virus. The growth of this virus was studied in cultures of tissues which, when maintained in a nutritionally deficient medium alone, lost their ability to support the growth of virus. The addition of beef embryo extract (BEE) to these cells restored this ability.

The enhancement of virus multiplication by BEE took place not only in cultures infected early, but also in cultures made deficient prior to infection. In the latter case, virus could remain in tissues for up to five days with no multiplication and still be induced to propagate.

It has been possible to show that the factors present in BEE responsible for this action are dialyzable and that the action does not depend entirely on a new population of cells. A chemically defined medium will duplicate the action of BEE. The implications of this study with reference to viral infections are discussed.

57. ELECTRON MICROSCOPY OF EARLY CYTOPLASMIC CHANGES DUE TO INFLUENZA VIRUS

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In order to study mechanisms by which respiratory epithelia become susceptible to bacterial infection, earlier observations were made of the effect of influenza virus on the bronchial epithelium of the mouse, and it was found

that ciliated cells persist in spite of severe infection. As a by-product, it was also found that cytoplasmic inclusion bodies are present in cells of the bronchial epithelium, and this finding was considered significant because inclusion bodies have not been widely recognized in the lesions due to influenza viruses. In the present work, advantage was taken of recently developed methods for electron microscopic visualization of cellular details in thin tissue sections. After fixation in buffered osmium tetroxide, the tissues were embedded in plastic, and sections were cut with glass knives at a thickness of approximately 0.05 micron. Study of the normal epithelium by this method showed that the internal fibers of cilia could be seen and sometimes counted. Microvilli were found on the surfaces of ciliated cells close to the cilia but easily distinguished from them. Mitochondria in these cells are filamentous but their cross sections appeared in these preparations as round or ovoid forms. Ergastoplasm (also called endoplasmic reticulum) was scanty in ciliated cells but abundant in nonciliated cells where it appeared as minute vesicular structures. In cells infected with influenza virus, the cytoplasmic inclusion bodies could be recognized easily, and they were found to contain many dense bodies having a size estimated to correspond with the known particle size of influenza virus. In ciliated cells of infected lungs, many curved linear structures were found.

The observations support the concept that these cytoplasmic inclusions consist at least partly of colony-like aggregations of virus. It seems likely also that the curved linear structures are abnormal forms of ergastoplasm caused by viral infection. Filamentous forms of influenza virus do not appear to be present in our preparations and these findings agree with the idea that influenza virus grows within the cytoplasm rather than on the surfaces of cells.

58. DISSECTION OF THE VECTORCARDIOGRAM (DIFFERENTIAL VECTORCARDIOGRAPHY)

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Vectorcardiograms obtained with the cathode ray oscilloscope in general have not been satisfactory because of photographic difficulties in distinguishing the individual characteristics of the component loops. Because the heartbeat is a repetitive cycle, the CR beam returns to the isoelectric point (origin of the vector loop) for a large part of the heart cycle, producing fogging in this area. In view of the importance of obtaining separated components of the vectorcardiogram, the authors have developed "an electrical dissector" (selective blanker or unblanker) which can be used with commercially available accessories: CRO, preamplifiers, ordinary camera, and a single channel direct writer electrocardiograph. Isolation of any part of the VCG loop is possible and a simultaneous record of the dissected portion is clearly marked on the direct writing electrocardiogram.

Unlike the original apparatus of Briller, Marchand, and Kossmann which was specially designed to be used with special CRO, camera, and three-channel direct recorder, our apparatus can be used with commercially available accessories. In addition we use the QRS spike as the trip voltage, allowing for a better signal-to-noise ratio than does the use of the P wave (Briller et al.).

Dissected vectorcardiograms have been obtained on several hundred patients with representative heart disease. Examples of such records will be presented, illustrating the intimate details of the origin and termination of the P, QRS, and ST-T loops.

59. BLOOD VOLUME, METHOD

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When total blood volumes determined by cell tagging methods are compared with those determined by plasma labelling methods, the results by the latter are higher. The explanation is that the hematocrit of venous blood, used in these determinations, is higher than the average body hematocrit. Venous blood with its greater number of cells contains a falsely larger number of tagged cells and a falsely diminished amount of tagged plasma. Accordingly, the results of the red cell methods of estimating total circulating blood volume are too low and of the plasma methods too high. However, venous blood samples coupled with venous blood hematocrits can be used in calculating true red cell volume with tagged cells, since in this instance the measurement is of the dilution of tagged cells within the circulating red cell mass. True plasma volume can be determined by using a plasma tagging substance and venous blood, since this measures the dilution of tagged plasma within the circulating plasma mass. The sum of the true red cell and plasma volumes is the true total circulating blood volume.

The separate, but simultaneous determination of red cell and plasma volumes is cumbersome and does not permit serial determinations. From a true plasma volume and an average body hematocrit, both a true red cell volume and total circulating blood volume can be calculated. The purpose of this paper is to show that the hematocrit of free-flowing finger blood approximates the average body hematocrit.

In 25 cases total blood volume was determined simultaneously with P^{32} tagged cells and I^{131} iodinated human serum albumin. From this data true red cell, plasma, and total circulating blood volumes were calculated. Total blood volume by the tagged cell method averaged 5 per cent lower and by the tagged plasma method 3 per cent higher than true blood volume. The finger hematocrit more closely approximated average body hematocrit than did venous hematocrit. Blood volume calculated by using true plasma volume and finger hematocrit averaged within 1.24 per cent of true blood volume. This procedure may easily be repeated serially for clinical studies.

The procedure and the significance of the findings will be discussed.

60. THE EFFECT OF RENAL FUNCTION UPON THE ELECTROCARDIOGRAPHIC RESPONSE TO ALKALOSIS

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Data presented by Darrow has demonstrated a shift of potassium from muscle cells and its excretion in the urine during an alkalosis. Several investigators have shown that infusion of sodium bicarbonate into animals results in hypokalemia and the electrocardiographic changes characteristic of potassium deficiency. However, existing data do not clarify the extent to

which these changes are dependent upon renal mechanisms or whether potassium may also migrate out of the cell in the absence of its renal excretion.

In the present study the effect of sodium bicarbonate infusion was compared in normal and nephrectomized or ureteral ligated dogs. Hypokalemia and the characteristic electrocardiographic changes occurred in three normal adult dogs under Nembutal injected with 0.9 Gm. of $NaHCO_3$ per kilogram in thirty minutes. Infusion of similar amounts of sodium bicarbonate into four dogs following nephrectomy or ureteral ligation produced no significant changes in the concentration of serum potassium and calcium. However, striking changes in the electrocardiographic pattern became apparent within thirty minutes after completion of alkali infusion, characterized by a marked elevation of the T waves and shortening of the Q-T interval. These electrocardiographic changes, resembling those seen in hyperkalemia, persisted throughout the six to eight hour period of observation. Injection of 15 mg. of calcium per kilogram intravenously into one animal failed to alter the electrocardiographic changes described above.

The foregoing observations indicate that the reduced potassium content of plasma and tissues in alkalosis is secondary to the increased renal excretion of potassium. Furthermore, the electrocardiographic changes associated with an alkalosis may not correlate with the concentration of serum electrolytes if there is a suppression of renal function. It may be presumed that the alkalosis has caused some change in the cells involved.

61. METABOLIC CHANGES IN HYPOTHERMIA

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Some investigators in cardiac surgery are utilizing hypothermia to permit longer periods of complete circulatory arrest; however, the high incidence of ventricular fibrillation has been discouraging. The present investigation of metabolic changes in the hypothermic dog was initiated to determine whether these changes contributed appreciably to the increased irritability of the myocardium during hypothermia. This was suggested by the observation that with hypoventilation the mortality due to ventricular fibrillation was 50 per cent, whereas with hyperventilation sufficient to maintain a blood pH above 7.6 the mortality was only 10 per cent. In hypothermia we have observed a fall in plasma potassium, phosphate, and glucose, no change in sodium, and a slight increase in chloride. The plasma volume (T-1824) generally decreased, the plasma protein either increased minimally or did not change, and there was a marked increase in the hematocrit. There was no change in the calculated extracellular volume, and the loss of total fluids averaged 5 per cent and appeared to be a function of the respiration. The potassium drop to about 75 per cent of normal occurred immediately after inducing hypothermia and remained at that level until warming. The blood glucose and plasma phosphate fell more gradually, and the maximum change varied in proportion to the fall in body temperature down to 25° C. The decrease in glucose and potassium could not be accounted for by loss in the urine. These changes have been studied to determine the influence of the following variables either alone or in combination; namely, hyperventilation, hypoventilation, cooling to 25° or 20° C., or complete circulatory arrest. In addition, the effects of administration of cortisone, high and low protein feeding, and exclusion of

the liver from the general circulation on phosphate, glucose, and potassium have been studied. Similar observations have been made in seven human subjects and the results will be compared with those obtained in dogs.

62. THE URINARY EXCRETION OF URACIL IN PERSONS WITH CHRONIC MYELOID LEUKEMIA

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(INTRODUCED BY WALTER H. PRITCHARD, M.D.)

Isolation and identification of substances present in the urine of persons with leukemia and not of normal subjects might lead to a better understanding of possible metabolic abnormalities associated with the abnormal cell growth and maturation in the leukemic organism. Studies with anion exchange column and paper chromatography of urine from two patients with chronic myeloid leukemia maintained on an animal protein free diet demonstrate the presence of a substance not found in normal urine under similar dietary conditions. This substance has been tentatively identified as uracil because of (1) similarity of ultraviolet spectrum, (2) similar chromatographic properties, both by anion exchange column and paper techniques, and (3) support of growth for a uracil-requiring yeast mutant *(Saccharomyces cerevisiae)*.

The metabolism of uracil, and other pyrimidines, in man is not known. However, previous studies by others indicate that pyrimidines are completely metabolized by mammals with excretion of their nitrogen as urea. Urinary excretion of the pyrimidine uracil by patients with chronic myeloid leukemia seemed inconsistent with this observation; therefore, the metabolism of uracil in the normal subject was investigated using chromatographic techniques. After feeding 10 Gm. of the pyrimidine to a normal person, three ultraviolet absorbing substances appeared in the urine. The first had spectral and chromatographic properties identical with uracil. The second was similar to uracil in its spectral and paper chromatographic properties, but differed in its activity on the anion exchange column. The third substance was excreted in small amounts, and from its spectral characteristics is thought to have been uracil conjugated at the 1- (3-) ring position with an as yet unidentified substance. This study would indicate that between 10 per cent and 20 per cent of the pyrimidine ring was recovered spectrophotometrically in the urine of the normal subject during three days after feeding 10 Gm. of uracil.

Uracil is a pyrimidine found in ribonucleic acid and not in desoxyribonucleic acid. Therefore, the urinary excretion of uracil, and not other pyrimidines, in persons with chronic myeloid leukemia suggests an association of abnormal ribonucleic acid metabolism with this disease.

63. DIURNAL RHYTHM OF THE SERUM IRON LEVEL: EFFECT OF DIET AND OF ENVIRONMENTAL TEMPERATURE

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(INTRODUCED BY C. J. WATSON, M.D.)

Numerous investigators have reported that the serum iron level varies throughout the day in a regular fashion, the higher values occurring in the morning and low values in the evening.

The results of this study showed that in subjects following their usual eating habits, i.e., three meals per day, this type of rhythm was noted. However, in subjects ingesting four iso-caloric, equally spaced meals, peak serum iron values occurred regularly in the afternoon or evening and low values in the morning. In other words, the diurnal curve was displaced so that the peak occurred six to eight hours later in the group on the four-meal regime than in those taking three meals at the usual times. The reasons for the displacement of this curve are not clear; various possibilities are discussed.

Maintenance of a constant temperature environment apparently did not influence the diurnal curve for serum iron values.

64. ORAL PHENYLBUTAZONE IN GOUT

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Oral phenylbutazone has been used in dosages of 100 or 200 mg. every four hours in 18 attacks of acute gout occurring in 15 patients. Most of the attacks were aborted in eight hours; one stopped in one and one-half hours and the longest in twelve hours. Subsidence of all joint discomfort occurred in twenty-four to forty-eight hours. No post therapy recurrences occurred. Phenylbutazone is an effective therapeutic agent in acute gouty arthritis. The abortion of the acute attack occurred before there was any demonstrable change in the serum or urine uric acid.

The uricosuric effect of oral phenylbutazone in dosages of 600, 800 and 1200 mg. per twenty-four hours on the serum uric acid and twenty-four hours urine uric acid excretion has been studied in 10 gouty and 6 nongouty arthritics. Phenylbutazone blood levels are available in 11 of the patients. A step-wise fall of serum uric acid to normal level occurred in all but two of the gout patients; one went into congestive failure and the other received a dosage of 600 mg. per day. The serum uric acid fell an average of 4.1 mg. per cent by the eighth day. There were no apparent differences between the groups who received 800 and 1,200 mg. of phenylbutazone per twenty-four hours. A lag of two or three days occurred before there was any appreciable fall in the serum uric acid. The excretion of uric acid was increased in all instances, except the patient with congestive heart failure. The increase was in a step-wise fashion and usually reached a maximum between the second and sixth day of therapy. The average increase of uric acid excretion per day in the gouty arthritics was 243.5 mg., and in the nongouty arthritics 177 mg.

The six nongouty arthritics behaved similar to the gout patients except that the average fall in uric acid was less; being 2.9 mg. on 800 to 1,200 mg. and 1.7 mg. on 600 mg. dosages of phenylbutazone.

Average phenylbutazone blood levels for dosages of 600, 800 and 1,200 mg. per twenty-four hours were 14.7 mg. per cent, 15.4 mg. per cent, and 20.4 mg. per cent, respectively.

65. AN ATTEMPT TO DETERMINE THE CRITICAL LEVEL OF BLOOD PRESSURE NECESSARY TO MAINTAIN GLOMERULAR FILTRATION RATE IN PATIENTS WITH HYPERTENSION BY THE USE OF GANGLIONIC BLOCKING AGENTS FOR REDUCING BLOOD PRESSURE

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Houston, Texas

(Introduced by John H. Moyer, M.D.)

With the widespread clinical use of therapeutic agents in the treatment of hypertension, it is desirable to determine a general range of blood pressure reduction to be used clinically which will maintain glomerular filtration rate and avoid impairment of renal function in patients with and without impaired renal function. Patients with hypertension were studied from the standpoint of glomerular filtration rate as related to mean blood pressure reduction when a ganglionic blocking agent (Pendiomide) was used intravenously. Glomerular filtration rate was determined by inulin clearance, and mean blood pressure was determined by intraarterial manometry. The mean blood pressure was lowered in a progressive manner approximately 20 mm. Hg at a time until maximum reduction was obtained for one hour or more. At each level the glomerular filtration rate was determined. Studies showed an initial reduction (average) in glomerular filtration rate which again increased after renal hemodynamic stabilization. When the mean blood pressure was reduced maximally for one hour or more, the average mean blood pressure showed a 38 per cent reduction. This represented an average mean blood pressure of approximately 100 mm. Hg, which is well within the normotensive range. At this point the average glomerular filtration rate was reduced about 35 per cent. It is concluded that in patients with hypertension, when the blood pressure is reduced there is a decrease of glomerular filtration rate initially as well as after a period of hemodynamic stabilization, which occurs within normotensive levels. There did not appear to be a consistent and predictable blood pressure level at which alterations in the glomerular filtration rates occurred.

66. THE EFFECT OF PEPPER AND DEPEPPERINIZED CONDIMENT UPON GASTRIC ACIDITY IN MAN

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Reports concerning the effect upon the gastrointestinal tract and particularly upon gastric acidity of commonly used condiments are sparse. References found in textbooks of gastroenterology, physiology, and pharmacology are few and equivocal, while in the current literature the problem is little discussed.

From the few reported observations the impression is gained that only a few condiments, e.g., paprika, pepper, mustard, and cinnamon, have an irritative effect on the gastric mucosa. Regarding the gastric secretory changes, only paprika seemed to increase the free acidity while mustard, if anything, decreased it. Nevertheless it was shown that the administration of spices to dogs receiving histamine in beeswax abetted the incidence of ulceration in them.

The above findings would suggest the advisability of withholding spices from the diet of patients with gastrointestinal disturbances, and, in fact, this

has been done customarily for many years. Many patients, however, dislike their dietary regimen without condiments, partly because of the lack of taste. Pepper in particular has been missed by some patients.

In view of the fact that the data on the effect of pepper on gastric acidity are meager, we are presenting the results of preliminary observations on the gastric acidity in ulcer patients who received 5 grams of pepper on one day and 5 Gm. of "depepperized" substance on the next day.

Fasting patients were intubated with a Levine tube. The gastric contents were completely aspirated and the amount, free and total acidity determined. At intervals of ten minutes, aspirations were continued until 4 specimens were obtained. Then the pepper or the depepperized substance was administered through the Levine tube and aspirations were continued for another hour at ten-minute intervals.

Results.—The observations to date have shown no rise in the free or total acidity in these patients following either substance. The curves seem to run fairly closely parallel. The findings thus far would support those who previously were unable to find any definite relationship between gastric acidity and pepper intake.

67. CARDIOPULMONARY EVALUATION DURING GRADED EXERCISE OF NORMALS AND PATIENTS WITH HEART AND LUNG DISEASE

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Sixty-five subjects ranging in age from 26 to 79 years were studied during graded exercise on a treadmill. The majority of the patients were cardiacs referred from the Cleveland Work Classification Clinic. Ballistocardiograms, resting electrocardiograms, vital capacity, and maximum breathing capacity were performed before exercise. During control, exercise, and recovery periods, bipolar chest lead electrocardiograms, respiratory efficiency, ventilatory equivalent, oxygen consumption, and respiratory rate were recorded. The exercise period consisted of walking at 1.7 miles per hour on a 10 per cent slope for ten minutes, or less if symptoms developed.

The normal response to this exercise was characterized by an increase of respiratory efficiency, decrease of ventilatory equivalent, respiratory rate less than 22 per minute, heart rate not exceeding 125 during exercise, lowering of the T wave, and an oxygen debt of less than 10 per cent. Fitness index (Bruce) was determined for all subjects.

Oxygen consumption during exercise varied with body weight within expected variations due to difference in muscular efficiency. Oxygen transport correlated with FI, and in all cases of FI below 10, oxygen transport was less than 6. There was good correlation between low FI and pulse in excess of 125 during exercise. Maximum breathing capacity and vital capacity were not related to FI in this series. The resting ballistocardiogram was related to performance in that 63.6 per cent of subjects with abnormal FI had abnormal ballistocardiograms. However, 44 per cent with abnormal ballistocardiograms had normal fitness indices.

The purpose of the study has been twofold: (1) to correlate fitness in everyday life with measured fitness on the treadmill and (2) to determine, if possible, means of dissecting heart from lung disease during periods of stress. We have noted exceptions to the correlation between the fitness index and everyday fitness. In such cases the requirements of everyday life and gainful occupation are less than those of the treadmill test. Although severe disease

of either the heart or lungs may be manifested by specific functional changes during exercise, it is often impossible to differentiate between the two with current techniques.

68. THE EFFECT OF ACTH ON ACUTE GLOMERULONEPHRITIS

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Eleven cases of acute glomerulonephritis, one case of subacute glomerulonephritis with persistent hematuria, and one case of chronic glomerulonephritis with acute exacerbation were followed by serial Addis counts of twelve-hour urine specimens. In addition, total eosinophile count and erythrocyte sedimentation rate were measured serially. Serial specific renal function tests were done in ten cases. Eight of these cases improved spontaneously within four weeks and this was associated with a precipitous drop in the 24-hour urinary excretion of red blood cells. Five cases showed persistent hematuria of 0.5 to 2.8 billion red blood cells per day for four weeks or longer. These five patients were treated with moderate doses of intramuscular ACTH, beginning with 60 to 80 units per day and decreasing to maintenance doses of 5 to 40 units per day. For all five cases there occurred a sharp fall in hematuria and a significant increase in TM_{PAH}. The renal plasma flow increased significantly in four cases and the glomerular filtration rate increased significantly in three. Recurrences of hematuria occurred in four cases and were correlated with a rise in the total eosinophile count, indicating inadequate ACTH therapy.

These studies indicate that ACTH in moderate dose may be of definite value in the treatment of acute glomerulonephritis with persistent hematuria; however, ACTH cannot be recommended as routine treatment because of the spontaneous improvement which occurs in the majority of patients.

69. THE ARTERIAL CIRCULATION IN A PATIENT WITH COMPLETE HEART BLOCK

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The stroke output and minute output of the heart in patients with complete heart block has been investigated by Alt, Walker, and Smith, and by Ellis and Weiss, and has been found to be within the normal range.

The present report deals with the measurement of the arterial circulation on five different occasions above the right elbow in a patient with complete heart block, probably on an arteriosclerotic basis. This study was made by means of a calibrated recording oscillometer with which it was possible to calibrate the amplitude of the recorded pulse wave in cubic centimeters. With this instrument, it is possible to record graphically the systolic and diastolic blood pressures as well as the arterial circulation at any chosen site. In this patient, the beat volume was uniformly increased, and the minute volume uniformly decreased above the right elbow. In five different studies, the minute volume at this site ranged between 53 and 76 ml. per minute (for normal adults this figure is from 80 to 120 ml.). The auscultatory and recorded systolic pressures were quite uniform, but the recorded diastolic pressure was about 20 mm. Hg above the auscultatory diastolic pressure which is in keeping with the literature. Also in this study it has been found that there is no correlation between the pulse pressure and the minute volume which is also in keeping with studies by other methods.

70. THE INFLUENCE OF MODIFICATION OF INTESTINAL FLORA UPON CHOLINE UTILIZATION

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(INTRODUCED BY HANS POPPER, M.D.)

According to previous investigations, in normal subjects more than 50 per cent of ingested choline is decomposed by intestinal bacteria to trimethylamine which is excreted in the urine. In liver disease this intestinal choline decomposition is reduced. The urinary trimethylamine excretion is not increased when choline is given intravenously nor when broad spectrum antibiotics are administered orally with choline. Also when large amounts of starch are fed trimethylamine formation from choline is significantly inhibited.

To study whether altering the intestinal flora by feeding of bacteria influences choline metabolism, a lactobacillus preparation (Bacid Culture) was fed for several days prior to ingestion of 4 Gm. of choline to 12 hospital control patients. These acid-producing lactobacilli were found not to attack choline in culture mediums. Total counts were made of stool before treatment for gram-negative rods, gram-positive cocci, anaerobic spores, yeasts, and molds, and anaerobic and aerobic lactobacilli; the urinary trimethylamine excretion was determined after choline feeding. The lactobacillus culture was fed at meal time for one week, and then during continued feeding the intestinal flora and the urinary trimethylamine excretion after feeding the test dose of choline were again examined. The feeding of lactobacillus culture reduced the number of gram-negative rods and gram-positive cocci by over 90 per cent with an accompanying rise of lactobacilli from a few hundred to several millions per gram of feces. The increase of the urinary trimethylamine excretion following the test dose of choline was reduced ranging from 27 per cent to 95 per cent (average 48 per cent) in comparison to the pretreatment period before alteration of the bacterial flora. It can be assumed that under such circumstances a much greater amount of administered choline is utilized. These findings emphasize the importance of the role of the intestinal bacteria in the utilization of choline.

71. AN ASSAY TECHNIQUE FOR EVALUATING THE EFFECT OF DIETARY CONSTITUENTS ON HYPERCHOLESTEREMIA

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(INTRODUCED BY EMMET B. BAY, M.D.)

Recently we have been interested in the ability of a cholesterol-free residue of mammalian brain to lower the serum cholesterol in cholesterol-fed chicks. In order to isolate the active fraction of this residue for human trials, a crude assay technique employing the serum cholesterol level of such birds was devised.

White Rock or New Hampshire cockerels were obtained at exactly 8 weeks of age and placed on a diet of starter mash, 1 per cent cholesterol, 10 per cent cottonseed oil, hereafter called the control diet. After two weeks on this diet, the birds were shifted to a new dietary mixture, similar to the control diet except that 5 or 10 Gm. of the chick starter mash was replaced by an equal weight of the dietary constituent to be investigated per 100 Gm. of

feed. The birds were fed 100 Gm. per day per bird, individually or in groups of three, and diet consumption measured. Serum cholesterol was determined on alar vein blood from each bird on the fifth and seventh days of each week. After a week on an experimental diet, the control diet was resumed for one week, after which experimental diets were reinstituted on alternate weeks until the bird reached 18 to 20 weeks of age. Beyond this period responsiveness, and especially the return to a rising cholesterol level, is variable and random fluctuations in serum cholesterol may occur. Illness, as indicated by loss of weight or appetite, will vitiate results.

A fairly consistent reduction of about 25 per cent in the level of serum cholesterol below that level anticipated from the control periods occurred on feeding the crude cholesterol-free, lipid-poor brain fraction as the experimental constituent. A cerebroside fraction, rich in phrenosin, was also active. Certain proteins, including casein, and mixed soybean sterols also had a hypocholesteremic effect, while dextrin, a pure carbohydrate, had a hypercholesteremic effect.

72. PRODUCTION AND REPAIR OF EXPERIMENTAL LIVER DYSFUNCTION IN MAN BY MODIFICATIONS OF DIETARY PROTEIN

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Recent investigations of kwashiorkor have given prominence to the relationship between chronic protein malnutrition and hepatic disease. In a study on the effect of dietary modifications of tryptophane intake in man, a diet relatively low in protein was fed to 31 men for periods from 365 to 458 days. Evidence of liver dysfunction was found in several subjects, while rats fed the same diet developed fatty livers. The abnormalities in both species were prevented or repaired by supplementing with protein. The basal diet for the men provided 2,300 calories, 6.5 Gm. of protein nitrogen, and 92 Gm. of fat. Food consumption was controlled at levels of 90 to 120 per cent of the basal diet depending upon the appetite of the individuals, as determined during a two-month preliminary period. Evidences of liver dysfunction which were repaired by protein suplementation included enlargement of the liver, abnormal retention of injected Bromsulphalein and abnormal levels of lactic and pyruvic acids in the blood after the oral administration of glucose. Supplementation of the deficient diet with 1 Gm. of lysine for 97 days, or 330 mg. of methionine for 97 days followed by 1.3 Gm. of choline for 59 days, or with 10 Gm. of lactalbumin for 159 days had little, if any, ameliorative effect. Supplementation with 30 Gm. of lactalbumin or feeding a "normal" diet providing 70 Gm. of protein/day was curative.

73. ADJUSTMENTS OF CORONARY FLOW TO THE WORK OF THE HEART

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WITH THE TECHNICAL ASSISTANCE OF EUGENE BOYD (BY INVITATION)

Total coronary flow has previously been determined in the isolated heart. In the open-chested animal, coronary flow has been partially measured primarily by cannulating one coronary artery or the coronary sinus. In the

present study, *total* coronary outflow was measured in the open-chested dog except for the small fraction draining into the left heart (Rodbard, Graham, and Williams, J. Applied Physiol., in press). The work of the left ventricle was varied by altering aortic resistance with a mechanical resistor, or by changing the amount of blood delivered by a pump. Measurements were made of coronary flow, left ventricular output, carotid and left auricular pressures, and oxygen contents of systemic arterial and coronary mixed venous blood.

Coronary flow, at steady states, varied from 7 to 40 per cent of the cardiac output. In addition to usual correlations, a three-dimensional plot was made for each experiment permitting evaluation of the independent effects of blood pressure and cardiac output on coronary flow. No correlation was observed between cardiac output and coronary flow except at high blood pressure levels. Thus, high output, unlike hypertension, fails to provide directly for the energy needs of the heart by increasing coronary flow.

At high pressures, high cardiac output was associated with increased coronary flow, at the same mean blood pressure. This phenomenon was not observed in experiments in which arterial hypoxemia was minimized, suggesting that high work levels in a hypoxic heart produce an active dilatation of the coronary bed. The presence of active dilatation under these conditions is further borne out by the disproportionate increase of coronary flow per increment of blood pressure at higher pressure levels. This dilatation is similar to that causing a progressive increase of coronary flow in experiments in which hypoxemia developed.

These results indicate that active coronary dilatation occurs whenever oxygen extraction from the blood and coronary flow at the given perfusion pressure are not sufficient for the oxygen needs of the heart. When active dilatation also becomes inadequate, the heart fails. Failure also occurs when coronary flow decreases following elevation of pressures in the right heart.

74. TREATMENT OF "ESSENTIAL HYPERTENSION" IN DOGS WITH RENIN AND ANTIRENIN

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Six dogs, three male and three female, with spontaneous (essential?) hypertension were each given daily intramuscular injections of semipurified renin from hog kidney cortex, for periods varying from four to nineteen months. Spontaneous hypertension in dogs is defined as a persistent mean blood pressure in excess of 140 mm. Hg as measured by direct femoral artery puncture, in the absence of obvious renal or other disease. Five dogs, each of which produced at least three to five units of antirenin to dog renin per ml. of plasma, showed a decrease in blood pressure to normotensive levels, without other physiologic changes or ill effects. These dogs showed greater antirenin titers to hog renin than to dog renin, whereas renal hypertensive dogs show the same titer. After treatment, there was a gradual increase in blood pressure which was correlated with a decrease in antirenin titer to 0. The sixth dog, which did not produce antirenin, developed a pressor response averaging 50 mm. Hg, with return to pretreatment blood pressure levels after therapy ceased. Control tissue extracts were without antihypertensive or pressor effects. Preliminary passive immunization experiments with antirenin suggest an antihypertensive effect correlated with the passively produced antirenin titer. These results constitute strong evidence for the role of renin (or

a closely related substance) in the pathogenesis of spontaneous hypertension in dogs. The possible relation of the latter to essential hypertension in man is deserving of thorough study and will be discussed.

75. THE LONG-TERM PROGNOSIS FOLLOWING MYOCARDIAL INFARCTION

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In the Heart Station files between 1932 and 1942, four hundred sixty-one cases were found of *first* acute myocardial infarction suitable for study. A complete follow-up was possible in 390 cases to the time of death or until Jan. 1, 1952. Of these 23 per cent died within the first two-month period. The remaining 77 per cent survived this initial period. Two-thirds of these surviving 285 patients lived over five years, two-fifths of them lived over ten years, and one-tenth lived over fifteen years. The average life expectancy of the 285 patients was slightly over eight years. This is longer than previously reported. The better prognosis found in the present study was due to two factors: (1) only patients with their first attack of myocardial infarction were included and (2) a large number were followed over five years.

There is a large group of the benign form of acute myocardial infarction with long-term survival, in addition to the immediately fatal cases and those surviving for only a year or so. Any new procedure, whether medical or surgical, claiming to prolong the life of surviving myocardial infarction patients must, therefore, be evaluated in terms of the more protracted expectancy now being revealed under the ordinary ceremonies of management.

An attempt was made to determine the factors affecting the immediate and long-term survival. Age at onset, pre-existing hypertension, angina pectoris, and diabetes mellitus were of importance in determining survival. So was the occurrence of "shock," congestive failure, and pulmonary embolism following the attack. Certain laboratory data also are helpful.

Almost two-thirds of the survivors were able to return to moderate or complete activity after convalescence. The best index to prognosis after recovery seems to be the ability to resume activity.

In 171 of the patients surviving over two months, the cause of death was known. In 94, it was due to a subsequent myocardial infarction; in 29, to congestive failure; in 31, to arteriosclerosis and its complications. Only 17 were attributable to noncardiovascular complications. The need is obvious, therefore, of establishing the mechanism of atherosclerosis and the factors that can be employed to prevent and treat it.

76. CLINICAL AND LABORATORY CORRELATION OF THE BALLISTOCARDIOGRAM

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(INTRODUCED BY C. L. SPURR, M.D.)

In view of the rapidly expanding clinical use of ballistocardiography the following observations on 600 hospitalized patients seem worthy of recording. The electromagnetic (Dock type) instrument containing a 70 microfarad condenser was used and tracings recorded by means of a Cambridge string

galvanometer. Records were made in the supine position during quiet respiration and while the breath was held in inspiration and in expiration. The criteria and grading system of Brown, Hoffman, and De Lalla were used.

Among 204 patients without clinical, electrocardiographic, or roentgenographic evidence of heart disease or conditions which might affect the cardiovascular system, 67 per cent of those under 40 years of age and 19 per cent of those over 40 years of age had "normal or borderline" ballistocardiograms. Grade 1 or 2 ballistocardiograms were observed in 25 per cent under 40 and 40 per cent over 40 years, and grade 3 or 4 ballistocardiograms noted in 9 per cent of those under 40 and in 41 per cent of those over 40 years of age. Among 42 patients with myocardial infarction (7 under 40 years of age) 5 per cent had "normal" ballistocardiograms, 19 per cent grade 1 or 2, and 76 per cent grade 3 or 4 tracings. Fourteen cases of essential hypertension presented the same type of distribution of ballistocardiograms as did the "normal" group. In a group of 46 patients with hypertensive heart disease, 70 per cent of whom were over 40 years of age, the following ballistocardiograms were noted: "normal," 12 per cent, grade 1-2, 33 per cent, and grade 3-4, 55 per cent. Thirty-six patients with valvular heart disease, symptomatic in most cases, presented the following types of ballistocardiograms: those under 40 years of age, 70 per cent "normal," 30 per cent grade 1 or 2, and 0 per cent grade 3 or 4; over 40 years of age, 19 per cent "normal," 42 per cent grade 1 or 2, and 39 per cent grade 3 or 4. Those patients having aortic insufficiency with or without other lesions and with or without failure all had "normal" or grade 1 ballistocardiograms with one exception who also had a myocardial infarction.

The effects of nitroglycerine and of changes in serum electrolyte concentration will be discussed. Of interest were two patients with coarctation of the aorta who demonstrated loss of characteristic ballistocardiogram alterations following nitroglycerine.

77. THE INDUCTION OF MYOCARDIAL LESIONS BY A COXSACKIE (PLEURODYNIA) VIRUS AND CORTISONE

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(INTRODUCED BY GEORGE E. BURCH, M.D.)

Coxsackie viruses are defined by their predilection for infant mice and hamsters and their inability to induce fatal disease in adults of these species. It has been demonstrated (Kilbourne and Horsfall, 1951) that the administration of cortisone to the adult mouse changes this state of intrinsic resistance to one of marked susceptibility terminating in death. Infection of the cortisone-injected adult mouse is attended by prolonged viremia associated with the presence of demonstrable virus in the liver, spleen, lung, brain, and pancreas. Injection of normal adult mice results in viral multiplication and pathologic changes exclusively confined to the pancreas (Pappenheimer and associates, 1951).

In the present investigation efforts have been made to adapt a strain of Coxsackie virus to the adult mouse by serial passage in the cortisone-injected host. The virus employed (tentatively classified as a Dalldorf Group B strain) was recovered directly in cortisone-injected adult mice from the stool of a patient with epidemic pleurodynia. Viral passages have been effected by the intraperitoneal inoculation of 10^{-2} brain suspensions in C.F.W. mice concomitantly injected with 5 mg. of cortisone acetate. On the eleventh

passage gross necrotic lesions of the myocardium were discovered by chance. Subsequent investigation of this phenomenon has adduced the following information:

1. Lesions are associated with the presence of virus in the myocardium.
2. Lesions are prevented by specific viral antiserum.
3. Cortisone is a requisite for the induction of gross lesions, but microscopically discernible myocarditis may occur in later passages with virus alone.
4. Small, focal areas of myocardial necrosis may be found in mice given cortisone, but no virus. Such lesions appear to differ qualitatively and quantitatively from those associated with the administration of virus.
5. Repetition of the initial and fourth viral passages with cortisone resulted in microscopically but not grossly evident myocarditis. It is thus probable that a degree of viral adaptation to the cortisone-injected adult has been accomplished by passage.

These experiments demonstrate that a hormone-viral synergism may be effected to induce devastating pathologic change in a "resistant" host.

78. THE MAXIMAL RENAL TUBULAR REABSORPTION OF FRUCTOSE IN NORMAL AND DIABETIC MAN

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The maximal tubular reabsorption of fructose (Tm_f) by the kidney has not been satisfactorily demonstrated in man. Using arterial blood samples, relatively stationary plasma fructose levels and sodium thiosulfate as the measure of glomerular filtration rate, we attempted to find this value. We gave hypertonic solutions of fructose to five diabetic patients, and to seven other persons without significant disturbance of their carbohydrate regulating mechanism or kidneys, and obtained plasma fructose levels up to 675 mg. per 100 mm. In most subjects plasma glucose levels steadily rose during infusion of fructose, reaching values of 240 to 357 mg. per cent glucose in the diabetics (none of whom had had insulin for twelve to twenty-four hours).

Below plasma fructose levels of 100 mg. per cent two normals reabsorbed fructose at rates of 38 to 118 mg./min./1.73 square meters of body surface area, while three diabetics reabsorbed fructose at 40 to 90 mg./min. At 100 to 200 mg. per cent of plasma fructose three normals reabsorbed fructose at rates of 40 to 92 mg./min. and three diabetics reabsorbed it at 56 to 92 mg./min. At levels of 200 to 300 mg. per cent three normals reabsorbed fructose at 60 to 102 mg./min. and four diabetics at 40 to 104 mg./min. In range of 300 to 675 mg. per cent plasma fructose five normal persons reabsorbed fructose at 40 to 130 mg./min. Except for two normals who had 26 and 81 per cent rises in fructose reabsorption as the serum fructose levels rose from 130 to 385 and 200 to 675 mg. per cent, respectively, there was no significant rise in reabsorption of fructose above plasma levels of 60 mg. per cent.

We conclude that the Tm_f in man varies from 40 to 130 mg./min. Saturation of all tubular reabsorptive mechanisms probably occurs at serum fructose levels under 100 mg. per cent in most normal persons. Diabetics have Tm_f values similar to normal persons and these are not altered at increased plasma glucose levels. Fructose is a potent osmotic diuretic at high plasma levels. At 675 mg. per cent plasma fructose one normal had a urine flow of 36.7 ml./min.

79. CLINICAL STUDIES OF AN ANTIEMETIC AGENT, CHLORPROMAZINE

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(INTRODUCED BY J. H. MOYER, M.D.)

Chlorpromazine (10-(γ-dimethylaminopropyl)-2-chlorophenothiazine hydrochloride) (S. K. F. 2601-A), in preliminary laboratory and clinical studies, exhibits antiemetic properties, presumably by central nervous system block of vomiting reflexes. This is a preliminary report on seventy-one patients with established nausea and vomiting who received chlorpromazine, 25 mg. to 50 mg. orally or parenterally. A majority received one to four doses. Fifty-four had excellent, fourteen good, one fair, and two poor, control of their nausea and vomiting. With variations from mild to marked degree, forty-one exhibited sedation, twenty-six dizziness, fifteen dry mucous membranes, sixteen tachycardia, fifteen hypotension.

Eighteen in this series received chlorpromazine for three to twenty-four days, with an average course being 7.5 days; frequency ranged from once daily to every three hours. The over-all control of nausea and emesis was: twelve excellent; five good; one fair. In varying degrees, fourteen exhibited sedation, six dizziness, three dry mucous membranes, seven hypotension. Three patients in the total series, receiving only one dose of chlorpromazine, exhibited significant side effects.

Eight additional normal adults without nausea and on no medications were given one 50 mg. dose of chlorpromazine. Seven exhibited sedation, three dizziness, two dryness of mouth, two slight hypotension. Six of these receiving 50 mg. intramuscularly and electrocardiographic studies immediately prior and one hour later exhibited no significant electrocardiographic changes.

80. STUDIES ON THE METABOLIC SIGNIFICANCE OF ACID-FASTNESS OF TUBERCLE BACILLI

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Acid-fastness of tubercle bacilli has great diagnostic and morphologic significance. Its metabolic implications, however, have not been fully studied. To investigate this problem the acid-fastness of tubercle bacilli was correlated with growth (nephelometry) and oxidative activity (tetrazolium reduction) in liquid medium.

During growth all tubercle bacilli are acid-fast and there is a constant relationship between the number of bacilli and tetrazolium reduction. In the resting phase bacilli remain acid-fast and, although growth has stopped, tetrazolium reduction continues and they always grow in subcultures. If streptomycin (50 μg/ml.) is added to the medium, growth and tetrazolium reduction stop a few days later. However, acid-fastness remains unchanged and the bacilli always grow in subcultures.

Isoniazid (10 μg/ml.) inhibits growth and tetrazolium reduction at a much slower rate than streptomycin, and parallel to the progressively de-

creasing growth and tetrazolium reduction the number of acid-fast bacilli decreases and of nonacid-fast bacilli increases. Seven to ten days after the isoniazid is added growth and tetrazolium reduction stop and none of the bacilli present are acid-fast. These nonacid-fast tubercle bacilli will not grow when subcultured.

In two experiments growth could no longer be measured eight days after the addition of isoniazid, but small amounts of tetrazolium continued to be reduced and from 5 to 20 per cent of the tubercle bacilli remained acid-fast. Subsequently growth and tetrazolium reduction increased again, and after twenty days only acid-fast isoniazid-resistant bacilli were found in the medium.

It was shown that the loss of acid-fastness under the influence of isoniazid occurs on solid medium as well as in liquid medium and in the presence or absence of Tween.

It would therefore appear that the loss of acid-fastness when exposed to isoniazid is a sign of metabolic death of tubercle bacilli, whereas retention of acid-fastness in bacilli exposed to isoniazid or streptomycin is associated with viabilty of such organisms.

81. AN ANATOMICAL STUDY OF THE CAUSES AND ASSOCIATED PATHOLOGY IN 673 AUTOPSIED CASES OF MASSIVE GASTROINTESTINAL HEMORRHAGE

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The number of reports of autopsy material concerning gastrointestinal hemorrhage has lagged far behind a rather voluminous clinical literature. Therefore, a study to determine the anatomical cause of hemorrhage was undertaken. The protocols of 28,400 consecutive autopsies at Cook County Hospital were reviewed. Massive gastrointestinal hemorrhage was found in 673 cases; in 310 cases it was the cause of death, and in 363 cases it was contributory or incidental to the cause of death. Correlating the clinical impressions with the findings at autopsy revealed the inaccuracy of present methods of diagnosis. Fatal hemorrhage occurred most frequently from esophageal varices. Gastric ulcers, the second greatest cause of fatal hemorrhage, occurred more often, bled more frequently and with greater severity than duodenal ulcers which was the next largest group. The fourth greatest cause of fatal hemorrhage was aneurysms rupturing into the gastrointestinal tract. Hemorrhage associated with carcinoma comprised the fifth group. The infrequency of fatal hemorrhage in often diagnosed lesions as diverticulitis, colitis, gastritis, and others was noted. In 25 cases no morphologic cause could be ascribed to the bleeding; these were considered parenchymatous in nature and were fatal in four instances.

Total bleeding was found in all ages including newborn infants. The greatest incidence of fatal hemorrhage occurred among the fifth, sixth, and seventh decades of life. There was a 4:1 predilection of the male sex and the white race.

The associated pathology was investigated in all bleeding cases and there appeared to be an increased incidence of hypertensive heart disease, coronary sclerosis, nephrosclerosis, and syphilitic aortitis from the amount usually ascribed to autopsy. In peptic ulcers the incidence of hepatobiliary disease

and goiterous lesions was increased. Other variations of associated pathology from the usual incidence were noted.

This basic anatomical approach to the problem of gastrointestinal hemorrhage may stimulate other similar correlations and helps to point out the marked variance between the clinical and morphologic diagnosis of gastrointestinal hemorrhage.

82. MECHANISMS OF INTERMITTENT VENTRICULAR BIGEMINY

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Bigeminy, i.e., the continuous alternation of short and long cycles, can result from a number of disturbances in either formation or conduction of the cardiac impulse. The present report deals only with bigeminy due to active ectopic impulse formation in the ventricles. By studying the factors that determine initiation, perpetuation, and intermittence of this form of bigeminy, it was hoped to gain insight into the mechanism of ventricular premature beats.

Analysis of 16 records from 13 patients with auricular fibrillation, and 15 records from 13 patients with sinus rhythm, led to the following classification of intermittent ventricular bigeminy.

A. Cases suggesting a *re-entry* mechanism. These were divided into Group I characterized by a fixed coupling in the presence of an irregular dominant rhythm (whether due to auricular fibrillation or sinus arrhythmia), and Group II characterized by progressive lengthening of coupling, in which the spacing of interectopic intervals did not support a parasystolic mechanism.

B. Cases with varying coupling in which the spacing of the ectopic beats suggested a *parasystolic mechanism*. These were divided into three groups. In Group I the interectopic intervals indicated a continuous parasystolic pacemaker. Intermittence of the resulting bigeminy was accounted for by interference in the ventricles, due to the normal refractory phase. In Group II the spacing of the ectopic beats indicated a continuous parasystole with intermittent exit block (exemplified by an instance in which this spacing showed the characteristic structure of Wenckebach periods). In Group III the intermittence was due to spontaneous intermittence of impulse formation in the parasystolic pacemaker, or temporary disappearance of its protection.

In a group of digitalis-induced intermittent bigeminy in patients with auricular fibrillation (Group A-I), careful analysis revealed a definite relationship between the appearance of the ectopic beats and the duration of the cycle preceding the beat to which the ectopic beat was coupled; a long cycle tended to initiate bigeminy whereas a short cycle tended to terminate or to preclude it. An explanation for this mechanism is presented and the criteria are given for the distinction from a similar pattern produced by aberrant ventricular conduction of supraventricular impulses. This differentiation is significant clinically.

83. THE EFFECT OF CHOLECYSTOSTOMY UPON SUBSEQUENT GALL BLADDER FUNCTION

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The material for this study consisted of 36 individuals who had had cholecystostomy for empyema of the gall bladder or acute cholecystitis. Stones were present in 27 instances at the time of the gall bladder drainage. Thirty-

two of the individuals were female. Cholecystograms were performed from four months to forty-three years after the cholecystostomy (average, 15.6 years).

Subsequent cholecystographic examination revealed normal visualization without calculi in 11 instances; there was normal visualization plus calculi in 10 instances. The gall bladder did not visualize in the remaining 15 instances; in 4 of these, radiopaque calculi were observed. Inasmuch as the majority of these individuals had operations because of acute inflammatory disease of the gall bladder, cholecystograms frequently had not been performed prior to surgery. In the seven instances in which cholecystograms performed before cholecystostomy were available for comparison with cholecystograms performed later (average, 10.6 years later), the results in the 2 cholecystographic studies were essentially the same. Ten of the 36 patients subsequently had cholecystectomy because of persistence of symptoms. These gall bladders, when removed, showed the changes of chronic cholecystitis and cholecystic fibrosis.

84. DEPRESSION OF GONADOTROPIN EXCRETION AS A METHOD FOR THE ASSAY OF ESTROGENS IN THE HUMAN SUBJECT

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Several clinical methods for assaying estrogens have been devised, in which criteria such as alleviation of menopausal symptoms or induction of "withdrawal bleeding" have been used for comparing estrogenic potency. Each of these procedures, however, has definite shortcomings and the need remains for methods which are objective and accurate and can be performed in readily available subjects.

Therefore, the ability of estrogens to decrease urinary gonadotropin excretion was studied in 21 postmenopausal or castrated women to determine if this property would afford a useful method for comparing estrogens in the human subject. The ovarian weight of immature female rats was used to determine urinary gonadotropin levels. Assays were performed before, and at approximately one-month intervals during therapy. Five oral estrogen preparations were studied: diethylstilbestrol; conjugated estrogens equine (Premarin); potassium estrone sulfate; estradiol; and potassium estradiol sulfate. Relatively small changes in estrogen dosage produced clear-cut differences in gonadotropin excretion. Whereas 0.5 mg. diethylstilbestrol daily produced a significant decrease in gonadotropin excretion in only one of six patients, 1.0 mg. produced definite suppression in each of seven patients. The other estrogens were less potent than diethylstilbestrol. Conjugated estrogens, equine was one-half, potassium estrone sulfate was one-fourth, and estradiol and potassium estradiol sulfate were one-fifth, as potent as diethylstilbestrol. These ratios are in general agreement with those obtained by workers employing other criteria for comparing estrogenic activity. It is concluded that the determination of the minimal amount of estrogen necessary to decrease the urinary gonadotropin excretion of postmenopausal or castrated women affords a method for comparing the potency of estrogens which is accurate and objective and can be performed in readily available subjects.

85. EFFECT OF ANTIBIOTIC USAGE IN THE HOSPITAL ON THE INCIDENCE OF ANTIBIOTIC-RESISTANT STRAINS AMONG PERSONNEL CARRYING STAPHYLOCOCCI

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Previous studies have indicated that when penicillin and the tetracycline antibiotics are administered to patients, a high incidence of strains of staphylococci resistant to these antibiotics develops among hospital personnel and is an important epidemiologic factor in the spread of these strains to patients and the community. In order to study the factors concerned with the development and perpetuation of such a reservoir, the impact of a new antibiotic "erythromycin" has been investigated. Erythromycin was substituted for penicillin in the treatment of almost all patients in one hospital for a five-month period. Tetracycline drugs were used as indicated. Near the end of the five-month period penicillin was reinstituted as the principal antibiotic used for treatment of patients.

During the five months in which erythromycin and the tetracyclines were used and penicillin restricted, the incidence of antibiotic-resistant strains of staphylococci among the carrier personnel changed as follows:

> Erythromycin at start of study, 0 per cent resistant, at the end of first month, 6 per cent resistant, second month, 25 per cent, third month, 50 per cent, fourth month, 64 per cent, fifth month, 70 per cent.
>
> Penicillin at start of study, 50 per cent, first month, 45 per cent, second month, 37 per cent, third month, 32 per cent, fourth month, 32 per cent, fifth month, 35 per cent.
>
> Aureomycin at start of study, 35 per cent, first month, 40 per cent, second month, 64 per cent, third month, 66 per cent, fourth month, 60 per cent, fifth month, 80 per cent.

Following the change from erythromycin back to penicillin with the tetracycline drug usage remaining the same, the incidence of antibiotic-resistant strains of staphylococci among the personnel changed as follows:

> Erythromycin at sixth month of study, 33 per cent, seventh month, 40 per cent, eighth month, 33 per cent, ninth month, 28 per cent.
>
> Penicillin at sixth month, 60 per cent, seventh month, 60 per cent, eighth month, 61 per cent, ninth month, 83 per cent.
>
> Aureomycin at sixth month, 60 per cent, seventh month, 60 per cent, eighth month, 75 per cent, ninth month, 58 per cent.

The factors concerned in establishing and maintaining carrier pools of resistant organisms include such factors as organisms brought in by the patient, percentage of patients receiving a given antibiotic, the development of resistant strains in treated patients, and the facility of spread from patients to personnel and personnel to patients.

(To be concluded in the December issue.)